Interactions of an Electron-Rich Tetracationic Tentacle Porphyrin with Calf Thymus DNA

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The interactions between the water-soluble tentacle porphyrin *meso*-tetrakis[4-[(3-(trimethylammonio)propyl)-oxy]phenyl]porphine (TθOPP) and calf thymus (CT) DNA had been found to exhibit unusual features: (a) At pH 7.0, TθOPP underwent extensive self-stacking along the DNA surface (Marzilli, *et al. J. Am. Chem. Soc.* **1992**, *114*, 7575); (b) the DNA-bound form of TθOPP was extensively protonated even at pH 7.0 (Pethö, *et al. J. Chem. Soc., Chem. Commun.* **1993**, 1547). In the studies presented here, the formation of the TθOPP−DNA adduct was investigated under various conditions of pH, salt concentration, [porphyrin]/[DNA base pair] ratio (R), and absence/presence of buffer. Under all conditions studied, TθOPP was an outside binder. Methods employed included circular dichroism (CD), UV−visible absorbance, and fluorescence spectrophotometry. Time-dependence experiments were also conducted in order to detect any change in binding characteristics over time. Evidence for outside-bound, self-stacked forms of TθOPP included a large conservative induced visible CD feature, whereas the evidence for DNA-bound, protonated TθOPP included a Soret band red-shifted to 451 nm, a single positive induced CD band at 451 nm, and a red-shifted fluorescence band at 740 nm. At least two forms of the outside-bound, self-stacked porphyrin were evident. At high R (0.25), hypochromicity of the Soret band and a conservative type CD feature indicated that TθOPP was extensively stacked. At lower R (0.05), increases in the Soret band intensity and changes in the band shape suggested a less extensively stacked form, but this form of TθOPP had a larger conservative feature in the CD spectrum. The intensity of the conservative band suggested that the less stacked form is very stable at $R = 0.05$. The CD data are consistent with a model in which the interacting TθOPP in the less stacked form has a different relative orientation than the more stacked form. Both high salt concentration and PIPES buffer appeared to stabilize the outside-bound, self-stacked form of TθOPP. At $R = 0.01$, a decrease in CD band intensity suggested that the porphyrin was less stacked than at $R = 0.05$; this change in stacking occurred over 1−2 h. Under these low R conditions where outside-bound self-stacking is less favored, the degree of TθOPP protonation is highest. The conservative-type CD spectrum changed to a simple positive CD band upon protonation of TθOPP, indicating that the binding mode underwent a transition from outside binding with self-stacking to simple outside binding. High salt concentration (100 mM NaCl) was found to decrease the degree of protonation, and the presence of PIPES buffer also decreased the degree of protonation at pH 7.0. Under conditions which favored protonation (low salt or no PIPES), the degree of protonation was found to increase gradually during the first few hours after sample preparation. Since in the time course of the experiment the total contents of the solution were unchanged, and since the isolated unstacked porphyrin should protonate quickly, these results suggest that stacking inhibits protonation of the DNA-bound porphyrin.

Introduction

The known ability of cationic porphyrins to associate with DNA and RNA has led to many studies of medical and biological applications of porphyrins. Recent applications are based on the antiviral[1,2] and anticancer[3−5] activities of porphyrins. *In vitro* structural studies of porphyrin−DNA adducts are important in order to gain greater knowledge of the factors which affect the biological activities of porphyrins. Furthermore, studies of synthetic porphyrins and their DNA binding modes have potential use in the development of therapeutic agents.

Cationic porphyrins have been found to associate with DNA in various binding modes.[6−8] The three major categories of porphyrin−DNA binding are intercalation, outside binding without self-stacking, and outside binding with self-stacking along the DNA surface.[6,9−12] Partial intercalation has also been suggested.[13,14] The binding mode or modes of a specific

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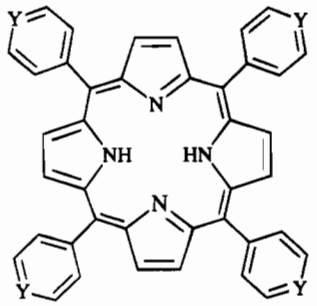
Chart 1



TθOPP: Y = C—O$(CH_2)_3N^+(CH_3)_3$

TθpyP: Y = N^+-$(CH_2)_3N^+(CH_3)_3$

TMAP: Y = C—$N^+(CH_3)_3$

TMpyP(4): Y = N^+-(CH_3)

porphyrin−DNA system are highly dependent on the substituent groups of the porphyrin and on the type of DNA. In order to achieve intercalation, it has been proposed that the porphyrin must have a limited effective thickness.[9,15,16] For example, the four planar *N*-methylpyridinium substituents of TMpyP(4)[17] (see ref 17 for abbreviations) allow intercalative binding,[9,15,18,19] whereas a similar porphyrin, TMAP, which has nonplanar *N*-trimethylammonium substituents, is an outside binder.[9,15] Previous studies have also indicated that porphyrins selectively intercalate in regions of DNA with a high percentage of GC base pairs and undergo outside binding in regions high in AT base pairs.[6,8,20] Since the base pairs are similar in size, this selectivity is unexpected on the basis of steric effects alone.

We have reported preliminary studies of the water-soluble "tentacle" porphyrins TθpyP and TθOPP (see Chart 1).[21,22] The porphyrins are of essentially identical size, bearing four propyl chains with a nonplanar *N*-trimethylammonium group at the end (the "tentacle" arms).[21] However, TθpyP and TθOPP differ in the electronic properties of their central core. The electron-donating ability of the phenoxy aromatic substituents of TθOPP leads to a pK_a for proton dissociation of the protonated species of 4.6 (1.8 μM TθOPP, 10 mM NaCl), a value much greater than that for TθpyP ($pK_a \approx 1$) with its pyridinium groups.[21] This high pK_a establishes TθOPP as having an electron-rich porphine core. Spectroscopic and viscometric studies indicate that TθpyP is an intercalator, whereas TθOPP is an outside binder with self-stacking along the DNA surface.[21] Other outside self-stacking porphyrins either bind in this fashion under limited conditions or, in some cases, can even intercalate under some conditions. Therefore, relatively little is understood about the DNA binding of self-stacked porphyrins. Such species could eventually provide useful information in the area of supramacromolecular structures.

Due to the relatively high pK_a of TθOPP, it is possible for the porphyrin to be bound to calf thymus (CT) DNA as the protonated species at physiological pH.[22] The protonated porphyrin exhibits DNA binding characteristics that are different from those of the free base.[22] This is a unique characteristic of this porphyrin and allows the comparison of a simple chemical process for a species unbound and bound to DNA.

The flexible tentacle arms of the porphyrin represent a unique characteristic for an outside binder. The tentacles potentially give TθOPP a relatively large "footprint" compared to other porphyrins, such that the outside-bound TθOPP is able to span several base pairs when bound in either an edge-on or face-on manner. McMillin and co-workers have suggested that outside groove binding produces a larger porphyrin footprint than intercalation.[23] Therefore, outside binding might be expected to be more stable at low porphyrin-to-DNA ratios, especially for porphyrins that are electron rich and have little tendency to intercalate. We extend this analysis to high ratios, where the self-stacked porphyrins should have a per-porphyrin footprint that is smaller than isolated edge-on or face-on bound porphyrins.

Here we present new findings extending our preliminary studies of the unusual porphyrin, TθOPP. Although it clearly is only an outside binder, our detailed study indicates that the DNA-binding characteristics of TθOPP have a complex dependence on pH, salt concentration, [porphyrin]/[DNA base pair] ratio, buffer, and time. We have therefore been able to show that there are several outside-bound, self-stacked forms of TθOPP.

Experimental Section

Materials. The base pair concentration of sonicated calf thymus DNA (~200 base pairs in length)[24] was determined by UV spectrophotometry ($\epsilon_{260} = 1.32 \times 10^4\ M^{-1}\ cm^{-1}$).[25] PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]), MES (*N*-morpholineethanesulfonic acid) (both from Sigma), sodium chloride, and diphenylthiocarbazone (both from J. T. Baker) were reagent grade and were used without further purification. Metal-free distilled water, used for all solutions, was prepared by exhaustive extraction with a carbon tetrachloride solution of diphenylthiocarbazone.[26] Sephadex LH-20 (25−100 μm) and Dowex-1 (mesh 100−200) were obtained from Sigma; other chemicals were used as received from Aldrich. All solvents were reagent grade.

Instrumentation. ^1H NMR spectra were recorded at 400 MHz (Varian VXR spectrometer). The UV−visible spectra were recorded at 25 ± 0.1 °C on a Varian Cary 3 UV−vis spectrophotometer with solutions in 1, 2, 5, or 10 mm quartz cuvettes. The CD spectra were recorded at ambient temperature on a Jasco 600 spectropolarimeter. Fluorescence studies were performed on a Spex Model F2T21I fluorescence spectrophotometer at 25 ± 0.1 °C. Excitation wavelengths were 418 nm (pH 7.0) and 445 nm (pH 2.0) in the absence of DNA and 425 nm in the presence of DNA. The pH of all samples was monitored with a 701A-type pH meter (Orion Research) and a combination glass electrode (Ingold Electrodes Inc.).

Synthesis of *meso*-Tetrakis[4-[(3-(trimethylammonio)propyl)oxy]-phenyl]porphine (TθOPP). To a solution of 5,10,15,20-tetrakis(4-

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hydroxyphenyl)porphyrin (0.2 g, 0.3 mmol) in dry DMF (100 mL) was added 1,3-dibromopropane (1.6 mL, 16 mmol) and K_2CO_3 (2.4 g, 17 mmol); the mixture was stirred at 40 °C under nitrogen for 3 days. The solvent was removed at 50 °C under high vacuum. The residue was taken up in $CHCl_3$ (50 mL) and washed with ice-water (3 × 20 mL). The organic layer was dried over $MgSO_4$ and filtered, the solvent removed *in vacuo*, and the residue dried under high vacuum (1 mmHg, 80 °C) to remove excess 1,3-dibromopropane. The crude material was dissolved in $CHCl_3$ (55 mL), and alumina was added to make a slurry. Concentration afforded a powder that was applied to an alumina column (4 × 45 cm) with benzene. The first major band was collected, the solvent was removed, and the residue was dried under vacuum (1 mmHg, 80 °C). The residue was crystallized by dissolving it in $CHCl_3$ (15 mL) and adding hexane (50 mL) to give the tetrakis{4-((3-bromopropyl)oxy)phenyl}porphyrin (TBPPP, 0.21 g, 60%): ^{1}H NMR ($CDCl_3$) 8.8 (s, 8H, *pyrrole*), 8.1 (d, 8H, *aromatic ring*), 7.2 (d, 8H, *aromatic ring*), 4.35 (t, 8H, OCH_2), 3.7 (t, 8H, CH_2Br), 2.45 ppm (m, 8H, $CH_2-CH_2-CH_2$). (Chemical shifts are downfield from internal tetramethylsilane). The UV−vis spectrum was similar to that reported previously by Milgrom for this compound.[27]

A dry tube with a screw cap was charged with a solution of TBPPP (10 mg) and an excess of trimethylamine (25% aqueous, 1 mL) in DMF (2 mL). The solution was allowed to stir vigorously at 60 °C for 3 days. The reaction solvents were removed, and the residue was dried under vacuum for 3 h. The solid material (~35 mg) was dissolved in MeOH/potassium phosphate buffer (10 mM, pH 7) (7:3) and purified twice on a Sephadex LH-20 column (2.5 × 30 cm) with the same solvent mixture. The first major band was collected, the solvents were removed under vacuum, and the residue was dried under vacuum. Purification of the entire product in this way gave the tetrapositively charged porphyrin, TθOPP (96 mg, 80%).

To prepare the chloride salt of TθOPP, a Dowex-1 (chloride form) anion-exchange resin column was prewashed with 0.1 N HCl and then washed with water until the eluate was at pH 7.0. The material from the Sephadex column was dissolved in water, loaded on the column, and eluted with water. This process was repeated twice. The water was removed *in vacuo* and the solid dried under vacuum. The final solid was obtained by dissolving it in MeOH and adding diethyl ether. ^{1}H NMR (CD_3OD): 8.90 (br. s, 8H, *pyrrole*), 8.12 (d, 8H, *aromatic ring*), 7.35 (d, 8H, *aromatic ring*), 4.32 (t, 8H, OCH_2), 3.81 (t, 8H, $CH_2N^+(CH_3)_3$, 3.65 (s, 36N, $N^+(CH_3)_3$), 2.43 ppm (m, 8H, $CH_2-CH_2-CH_2$). Acetone was used as an internal reference; chemical shifts are downfield relative to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (Aldrich).

Methods. For sample preparation, a 10 μL aliquot of 1.5 mM TθOPP stock solution was diluted in 2.00 mL of buffer or NaCl solution. Then, an aliquot of 47.0 mM CT DNA was added to the dilute TθOPP solution so that R ([porphyrin]/[DNA base pairs]) = 0.25, 0.05, or 0.01. In the salt effect and buffer effect titration experiments, the pH was measured and re-adjusted to the desired value (6.0 or 7.0) after each addition of titrant. The UV−vis, CD, and fluorescence spectra were found to be time-dependent and therefore were monitored with time. When the spectra were not being recorded, the solutions were stored in the dark at room temperature.

Results

UV−Visible Spectroscopy. Studies with No DNA. At pH 7.0, an aqueous solution of TθOPP (designated as **P** when only two of the central nitrogens are protonated) appears red with a Soret band (So) at 418 nm and a small shoulder (sh) at ~396 nm (Figure 1). For 2.0 μM TθOPP (pH 7.0), the ϵ_{So} and ϵ_{sh} values are 5.5 × 10^5 and 1.0 × 10^5 M^{-1} cm^{-1}, respectively (units for molar absorptivity will not be repeated). According to the expected D_{2h} symmetry of TθOPP (**P**), four Q bands should be present. Bands at 520, 558, and 640 nm and a shoulder on the 558 nm band at ~583 nm are assigned to the

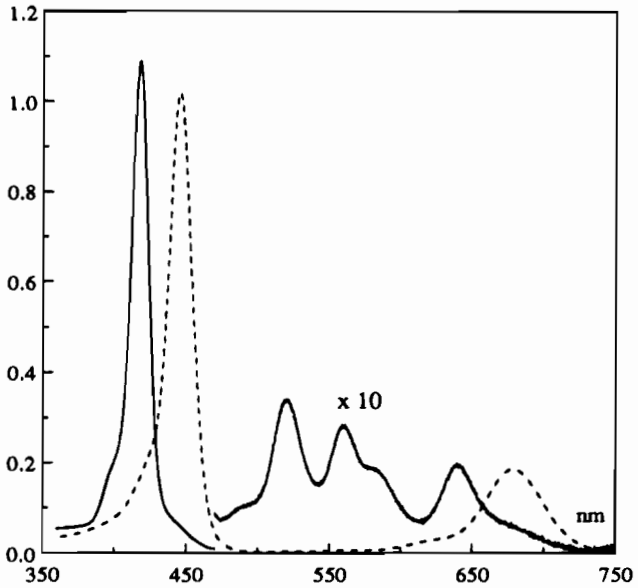

Figure 1. Visible absorption spectrum of 2.0 μM TθOPP at pH 7 (—) and pH 2 (- - -). Spectra were obtained at 25 °C.

$Q_y(1,0)$, $Q_y(0,0)$, $Q_x(0,0)$, and $Q_x(1,0)$ bands, respectively, on the basis of previous porphyrin assignments.[28,29]

The color of an acidic solution of TθOPP (2.0 μM, pH 2.0) is green, typical of protonated *meso*-substituted porphyrins.[30] The Soret band is red-shifted to 445 nm (ϵ_{445} = 5.1 × 10^5) upon protonation (Figure 1). For the protonated TθOPP, two Q bands are present; the strongest Q band has a λ_{max} of 680 nm, whereas the other appears as a shoulder at ~620 nm. The reduction in the number of Q bands from four to two is consistent with the production of the diprotonated form of TθOPP (**H_2P^{2+}**).[29] A 1.8 μM solution of TθOPP was titrated from pH 7.0 to 2.0 in order to determine the pK_a(s) of the protonated species. The protonation reaction was monitored by the increase in the intensity of the 445 nm band with decreasing pH. The titration did not show a clean isosbestic point (~430 nm), suggesting that this is not a simple $P + 2H^+ \rightleftharpoons H_2P^{2+}$ equilibrium. The pH at 50% conversion of the free base to the protonated porphyrin (1.8 μM TθOPP in 10 mM NaCl) was determined to be 4.6. Similar titrations with various TθOPP and salt concentrations consistently gave values of 4.5−4.8, even though the intensity of the protonated band was highly dependent on the TθOPP and salt concentrations. For a dication, the pH value at 50% conversion approximates $^1/_2$ of the p$K_{3,4}$ value, where $K_{3,4}$ is the equilibrium constant for the conversion of the dication to the free base; for a monocation, this value approximates pK_3 (conversion of monocation to free base). It remains unclear whether the observed protonated TθOPP is the monocation or the dication. Equilibrium studies which might have distinguished between a mono- or diprotonation process[31,32] were unsuccessful due to the complicated behavior of TθOPP. Specifically, the degree of protonation was found to be highly dependent on salt concentration, suggesting that self-aggregation of the porphyrin has an effect on protonation. In addition, strong adsorption of TθOPP on the cuvette surface precluded studies at low porphyrin concentrations, where self-aggregation would presumably be reduced.

In titration of porphyrins without a positive charge directly in the tetraphenyl ring structure itself, it is common to observe direct production of **H_2P^{2+}** with the absence of the monocation

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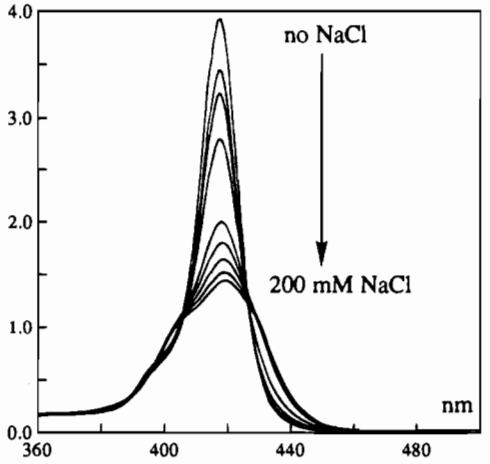

Figure 2. The effect of increasing NaCl concentration (0–200 mM) on the visible absorption spectrum of 7.3 μM TθOPP. Values used were 0, 5, 10, 20, 50, 70, 100, 150, and 200 mM NaCl.

(**HP**$^+$).[32,33] However, because the structure of the protonated species cannot be established conclusively, the protonated form of TθOPP will be referred to as **HP**, with no specific charge or number of protons indicated.

The addition of aliquots of a NaCl solution to a 7.3 μM TθOPP solution (pH 7.0, no buffer) caused an incremental decrease in ϵ_{So} (Figure 2). At 10 mM NaCl, ϵ_{So} was reduced to 4.5×10^5, and at 200 mM NaCl, the Soret band was very broad with $\epsilon_{So} = 1.9 \times 10^5$. The ratio of the intensities of the Soret band and the shoulder (A_{So}/A_{sh}) decreased with increasing [NaCl]; this ratio is a useful indicator for stacking.

The addition of PIPES buffer into 7.5 μM TθOPP (pH 7.0, 10 mM NaCl) had a similar effect on the Soret band (data not shown). At 1 mM PIPES, ϵ_{So} decreased from 4.5×10^5 to 4.0×10^5, while, at 10 mM PIPES, ϵ_{So} decreased further to 2.7×10^5. The A_{So}/A_{sh} ratio was 4.0 and 1.9 at 1 and 10 mM PIPES, respectively.

Studies with CT DNA. The pK_a of **bHP** (protonated TθOPP bound to CT DNA) was determined at $R = 0.01$ (10 mM NaCl) as described above (R = [porphyrin]/[DNA base pairs]). Interestingly, the irregular behavior of TθOPP alone in solution is reduced in the presence of DNA. The pK_a of **bHP** was 6.6–6.8, approximately two pH units higher than for the unbound form (pK_a = 4.6). For a DNA-bound species, this type of pK_a increase, which has been rarely observed, can be attributed to the polyanionic environment of the DNA backbone.[34] In the pK_a determination of the bound form, the isosbestic point at ~435 nm was well-maintained (Figure S1). Whereas the Soret band λ_{max} value of unbound, protonated TθOPP had been 445 nm, **bHP** had a red-shifted λ_{max} of 451 nm. The molar absorptivity of this band is designated as ϵ_{bHP}.

pH 7.0. The results of the TθOPP–CT DNA UV–vis studies are included in Table 1. Hypochromicity (H) is defined here as $[(A_o - A_s)/A_o] \times 100\%$, where A_o and A_s are the absorbances at the Soret λ_{max} without and with added electrolytes, e.g. DNA, respectively. In a 7.5 μM TθOPP solution (10 mM PIPES, 10 mM NaCl), the wavelength of the Soret maximum (λ_{So}) was red shifted by 6–7 nm upon addition of DNA (Figure 3a). The ϵ_{So} values were similar for all three R values, with a slight increase in ϵ_{So} with decreasing R. In addition, the A_{So}/A_{sh} ratio was 1.2 for $R = 0.25$ and increased with decreasing R. This increasing ratio is due to the shoulder at ~410 nm undergoing a slight red shift (~5 nm) with increasing DNA concentration, causing the shoulder to become less pronounced. At $R = 0.05$, a weak band at ~451 nm (**bHP**) was present which was not observed at $R = 0.25$. At $R = 0.01$, the **bHP** band had the same intensity as at $R = 0.05$. Changes for these spectra over the course of 1 day were negligible, except for a small increase in the **bHP** band at $R = 0.01$. The essential spectral features were consistent through all experiments, and most results were reproducible within 10% with different preparations of CT DNA and different stock solutions of TθOPP. The most variable results involved the intensity of the **bHP** band in experiments using different TθOPP stock solutions.

In view of the dependence of the TθOPP spectrum on NaCl concentration, the study above was repeated with 100 mM NaCl (10 mM PIPES). As shown in Figure 3b and in Table 1, λ_{So} was 424 nm for all three R values. The ϵ_{So} values were smaller than those obtained in 10 mM NaCl at $R = 0.25$ and 0.05 but were the same at $R = 0.01$. The A_{So}/A_{sh} ratios observed in 100 mM NaCl at $R = 0.25$ and 0.05 were also slightly smaller than those in 10 mM NaCl but the same at $R = 0.01$. At 451 nm, no hint of a band was found at any of the R values studied, in contrast to the results at 10 mM NaCl. Only slight spectral changes were observed over time for all three solutions.

To determine the effect of the buffer, a DNA addition experiment was performed in the absence of PIPES (7.5 μM TθOPP, pH 7.0, 10 mM NaCl, Figure S2). The λ_{So} and ϵ_{So} values were similar to those in the presence of PIPES (Table 1). However, for the $R = 0.05$ and 0.01 solutions, the **bHP** band was significantly larger (Figure S2) than in the presence of PIPES (Figure 3a). The absorptivity of the **bHP** band increased over 1 day (Figure S2), and the color of the solution became green, the same color that the porphyrin has in water at pH 2. In contrast to the results in the presence of PIPES, the **bHP** band in the absence of PIPES was significantly larger and underwent larger time-dependent intensity increases than in the presence of PIPES. These data indicate that PIPES decreases the extent of protonation at pH 7.0.

pH 6.0. Solutions at pH 6.0 were investigated to verify that the 451 nm band found at pH 7.0 was attributable to **bHP**. At $R = 0.25$ (10 mM MES, 10 mM NaCl), the λ_{So} and ϵ_{So} values were similar to those at pH 7.0 (Figures 3a and 4a). The **bHP** band at 451 nm was present as a shoulder, whereas at pH 7.0 this band had not been apparent at $R = 0.25$. The absorptivities remained the same for the entire spectrum over 24 h. At lower R values, large **bHP** bands were present. At $R = 0.05$, ϵ_{So} decreased by 13% and ϵ_{bHP} increased by 30% over 24 h. At $R = 0.01$, ϵ_{So} was smaller by ~15% and ϵ_{bHP} was larger by ~25% than at $R = 0.05$. One day later, ϵ_{bHP} had increased further to 3.9×10^5. These results indicate a larger amount of **bHP** at pH 6.0 than at pH 7.0 and, at low R, an increase in the amount of **bHP** with time. Additionally, in the Q-band region of the spectra, the **bHP** bands (data not shown) were of the same shape as those of the **HP** species (Figure 1), except they were red-shifted by 8 nm.

A pronounced NaCl effect was found at pH 6.0. In 100 mM NaCl (10 mM MES) at $R = 0.25$, there was no band at 451 nm (Figure 4b), and ϵ_{So} was 10% larger than in 10 mM NaCl (Table 1). The A_{So}/A_{sh} ratio was only slightly less than in 10 mM NaCl, even though in the absence of CT DNA the spectrum had been much broader in 100 mM NaCl and the A_{So}/A_{sh} ratio significantly lower (1.7 vs 5.3 in 10 mM NaCl). At $R = 0.05$, ϵ_{bHP} was 1.1×10^5, a value less than half that in 10 mM NaCl, and was unchanged over the course of 1 day. At $R = 0.01$, ϵ_{So} was smaller by ~5% than at $R = 0.05$ but was significantly larger

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(34) Scheek, R. M.; Zuiderweg, E. R. P.; Klappe, K. J. M.; van Boom, J. H.; Kaptein, R.; Rüterjans, H.; Beyreuther, K. *Biochemistry* **1983**, *22*, 228–235.

Table 1. UV−Visible Characteristics of TθOPP in the Presence of CT DNA

	immediately after mixing					24 h after mixing				
R	λ_{So} (nm)	ϵ_{So} (M^{-1} cm^{-1})	H (%)	A_{So}/A_{sh}	ϵ_{bHP} (M^{-1} cm^{-1})	λ_{So} (nm)	ϵ_{So} (M^{-1} cm^{-1})	H (%)	A_{So}/A_{sh}	ϵ_{bHP} (M^{-1} cm^{-1})
					10 mM PIPES/10 mM NaCl at pH 7.0					
0.25	424	2.1×10^5	23	1.2		425	2.0×10^5	24	1.2	
0.05	424	2.3×10^5	12	1.3	3×10^4	425	2.4×10^5	9	1.3	3×10^4
0.01	425	2.4×10^5	6	1.4	3×10^4	426	2.5×10^5	4	1.4	4×10^4
					10 mM PIPES/100 mM NaCl at pH 7.0					
0.25	424	1.8×10^5	16	1.1		425	1.6×10^5	25	1.1	
0.05	424	2.1×10^5	1	1.2		425	2.0×10^5	6	1.2	
0.01	424	2.4×10^5	−13	1.4		425	2.4×10^5	−13	1.4	
					No Buffer/10 mM NaCl at pH 7.0					
0.25	423	2.0×10^5	45	1.3		424	2.0×10^5	46	1.3	
0.05	423	2.3×10^5	35	1.3	5×10^4	425	2.3×10^5	36	1.4	8×10^4
0.01	424	2.2×10^5	39	1.4	9×10^4	425	2.0×10^5	43	1.6	1.5×10^5
					10 mM MES/10 mM NaCl at pH 6.0					
0.25	424	2.0×10^5	54	1.3	9×10^4	424	2.0×10^5	54	1.3	9×10^4
0.05	426	1.5×10^5	65		2.7×10^5	426	1.3×10^5	70		3.5×10^5
0.01	427	1.3×10^5	71		3.4×10^5	427	1.2×10^5	73		3.9×10^5
					10 mM MES/100 mM NaCl at pH 6.0					
0.25	424	2.2×10^5	20	1.2		424	2.0×10^5	26	1.2	
0.05	424	2.1×10^5	22	1.3	1.1×10^5	425	2.0×10^5	27	1.3	1.1×10^5
0.01	425	2.0×10^5	26	1.4	1.5×10^5	425	2.0×10^5	26	1.4	1.4×10^5
					No buffer/10 mM NaCl at pH 6.0					
0.25	424	1.9×10^5	58	1.3	1.0×10^5	425	2.0×10^5	58	1.3	1.0×10^5
0.05	426	1.6×10^5	66		2.4×10^5	427	1.3×10^5	72		3.5×10^5
0.01	427	1.4×10^5	70		2.8×10^5	427	1.1×10^5	77		3.9×10^5

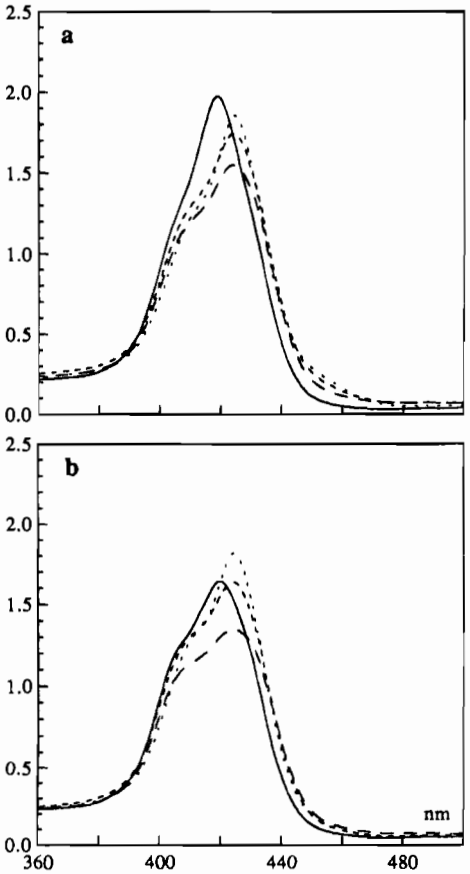

Figure 3. Visible absorption spectrum of 7.5 μM TθOPP at various porphyrin/DNA base pair ratios: No DNA (—); $R = 0.25$ (— —); $R = 0.05$ (— —); $R = 0.01$ (---), (a) solutions contained 10 mM PIPES buffer (pH 7.0), 10 mM NaCl; (b) solutions contained 10 mM PIPES buffer (pH 7.0), 100 mM NaCl. Spectra were acquired immediately after addition of DNA.

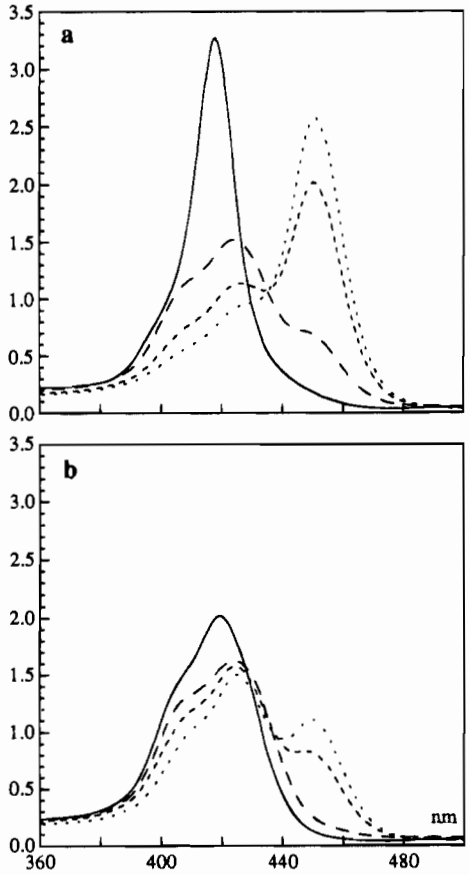

Figure 4. Visible absorption spectrum of 7.5 μM TθOPP in the presence of CT DNA: No DNA (—); $R = 0.25$ (— —); $R = 0.05$ (— —); $R = 0.01$ (---); (a) solutions contained 10 mM MES buffer (pH 6.0), 10 mM NaCl; (b) solutions contained 10 mM MES buffer (pH 6.0), 100 mM NaCl. Spectra were acquired immediately after addition of DNA.

than in 10 mM NaCl. Also, ϵ_{bHP} was more intense by ~30% than at $R = 0.05$ but less intense by ~50% than in 10 mM NaCl. In the $R = 0.01$ spectrum, ϵ_{bHP} decreased by ~7% after 1 day. These results suggest that the high salt concentration caused a decrease in the amount of **bHP** relative to the amount of bound, unprotonated TθOPP (**bP**).

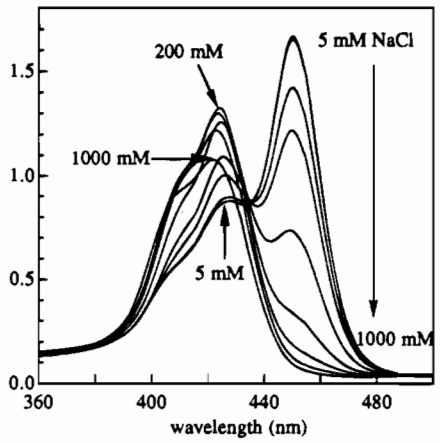

Figure 5. Visible absorption spectrum of 7.5 μM TθOPP during NaCl titration of TθOPP/CT DNA adduct (R = 0.05). NaCl concentration range: 5−1000 mM; pH = 6.0; no buffer; T = 25 °C. Maximum absorbance of the 424 nm peak occurs in 200 mM NaCl.

As shown in Figure S3, in the absence of MES (pH 6.0, 10 mM NaCl), the visible spectral results were only slightly different from those in the presence of MES. At R = 0.25, ϵ_{So} was 5% less than the value in 10 mM MES; ϵ_{bHP} was ~10% greater than with MES. After 1 day, ϵ_{So} increased by 5%, but ϵ_{bHP} remained the same. At R = 0.05, ϵ_{So} was slightly greater than the value in 10 mM MES, and ϵ_{bHP} was 10% smaller than in the buffer. However, after 1 day ϵ_{bHP} underwent a large increase. Consequently, ϵ_{bHP} was exactly the same in the absence of the buffer after 1 day as in 10 mM MES after 1 day. At R = 0.01, ϵ_{bHP} was 20% less than that found in 10 mM MES. After 1 day, ϵ_{bHP} was identical to the value in buffer (3.9×10^5). In summary, soon after sample preparation, the ϵ_{bHP} values were 10−20% less than those in 10 mM MES, and after 1 day they were equal to those in 10 mM MES.

The R = 0.01 spectrum after 1 day (pH 6.0, 10 mM NaCl) contained a significant shoulder in the Soret region (at 425 nm). To explore whether this band is a result of residual amounts of **bP** or a shoulder on the 451 nm band of **bHP**, we obtained the spectra of identical solutions (R = 0.01, no buffer, 10 mM NaCl) at pH 5.5, 5.0, and 4.5 (spectra not shown). The shoulder reached its minimum height ($\epsilon_{425} = \sim 1.0 \times 10^5$) at pH 5.0, indicating that, at pH 5.0 and below, all of the bound TθOPP is protonated and that the **bHP** band has a shoulder at ~425 nm.

The effect of NaCl on the TθOPP−DNA complex was probed in more detail by titrating NaCl into a solution of 7.5 μM TθOPP (R = 0.05, pH 6.0, no buffer, 10 mM NaCl). As shown in Figure 5, at the first titration point (5 mM NaCl), the **bHP** band was the largest band in the spectrum. With increasing [NaCl], this band decreased in intensity until it became an unresolved shoulder of the emerging 424 nm band at 200 mM NaCl and disappeared at higher [NaCl]. As the **bHP** band decreased, the Soret band which is characteristic of **bP** increased in intensity and exhibited maximum absorbance at ~200 mM NaCl (λ_{max} = 424 nm, $\epsilon_{424} = 1.9 \times 10^5$). With increasing NaCl concentration above 200 mM, the Soret band decreased in intensity and blue-shifted to 422 nm at 1000 mM NaCl.

Circular Dichroism. pH 7.0. The binding of TθOPP to CT DNA was also monitored through circular dichroism (CD) measurements. The overall shape of the CD spectrum was independent of the NaCl concentration or the presence or absence of PIPES buffer. In addition to a small positive feature {+s} in the region between 400 and 410 nm, the spectra also contained a conservative, exciton-type feature centered at ~430 nm, with the negative component {−exc} appearing between

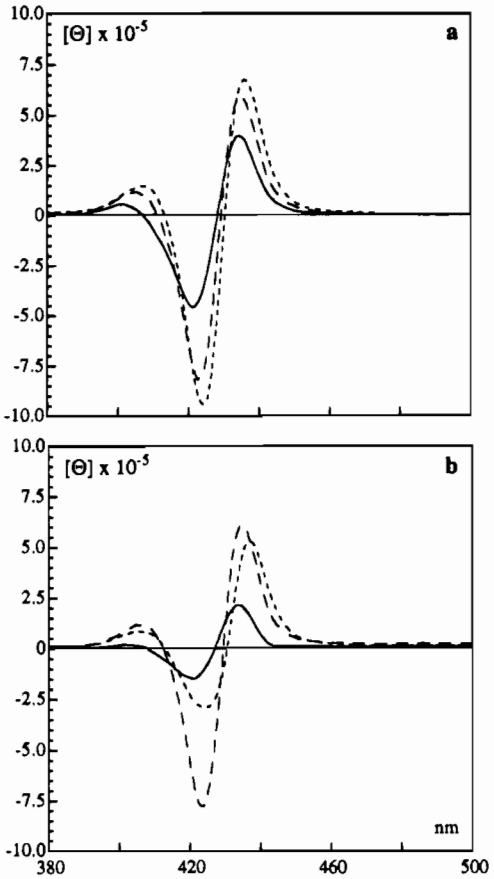

Figure 6. Circular dichroism spectrum of 7.5 μM TθOPP at various porphyrin/DNA base pair ratios: (a) Immediately after mixing; (b) 24 h after mixing. Solutions contained 10 mM PIPES buffer (pH 7.0), 10 mM NaCl. R = 0.25 (—), R = 0.05 (— —), and R = 0.01 (- - -). Units for [Θ] are deg cm^2 dmol^{-1}.

420 nm and 425 nm and the positive component {+exc} appearing between 435 and 438 nm (see Figure 6). Generally, the order of molar ellipticity ([Θ]) (omitting the sign) was $[\Theta]_{-exc} > [\Theta]_{+exc} > [\Theta]_{+s}$. The λ_{max} and [Θ] values depended mainly on R but also on the concentrations of TθOPP and NaCl. In many cases, the [Θ] values were time-dependent.

Table 2 contains the results of the TθOPP−CT DNA experiments as monitored by CD. In 10 mM NaCl (7.5 μM TθOPP, 10 mM PIPES) at R = 0.25, the [Θ] values were 6×10^4, -4.6×10^5 and 4.0×10^5 deg cm^2 dmol^{-1} (units for molar ellipticity will not be repeated) for {+s}, {−exc}, and {+exc}, respectively (Figure 6). These values decreased by 67%, 67%, and 45%, respectively, after 1 day. At R = 0.05, the λ_{max} values for the +s and −exc bands were slightly red-shifted, while the +exc band did not shift. The [Θ] values at R = 0.05 were approximately 2-fold larger than at R = 0.25 for all three bands. For R = 0.01, the initial spectrum had 15−20% larger ellipticities than in the initial R = 0.05 spectrum. Also, the R = 0.01 +s, −exc, and +exc bands were all slightly red shifted with respect to the R = 0.05 spectra. After 1 day, the ellipticities of the three bands had decreased by 40, 69, and 21%, respectively, so that the [Θ] values at R = 0.01 were smaller than at R = 0.05 but larger than at R = 0.25.

At the higher [NaCl] of 100 mM, for R = 0.25, the intensities in the initial spectra in 100 mM NaCl were ~50% smaller than in 10 mM NaCl (Figure S4a). The molar ellipticities of the −exc and +exc bands underwent approximately 2-fold increases over the course of 1 day (Figure S4b), and the +s band increased by more than 6-fold. Also after 1 day, the λ_{max} values for the three bands all shifted to shorter wavelengths by 1−4 nm, and

Table 2. Circular Dichroism Characteristics of TθOPP in the Presence of CT DNA

	immediately after mixing								24 h after mixing							
R	λ_{+s} (nm)	$[\Theta]_{+s}$ ($\times 10^{-5}$)	λ_{-exc} (nm)	$[\Theta]_{-exc}$ ($\times 10^{-5}$)	λ_{+exc} (nm)	$[\Theta]_{+exc}$ ($\times 10^{-5}$)	λ_{bHP} (nm)	$[\Theta]_{bHP}$ ($\times 10^{-5}$)	λ_{+s} (nm)	$[\Theta]_{+s}$ ($\times 10^{-5}$)	λ_{-exc} (nm)	$[\Theta]_{-exc}$ ($\times 10^{-5}$)	λ_{+exc} (nm)	$[\Theta]_{+exc}$ ($\times 10^{-5}$)	λ_{bHP} (nm)	$[\Theta]_{bHP}$ ($\times 10^{-5}$)
						10 mM PIPES/10 mM NaCl at pH 7.0										
0.25	401	0.6	421	−4.6	435	4.0			401	0.2	421	−1.5	433	2.2		
0.05	405	1.2	423	−8.2	435	5.9			405	1.2	423	−7.8	435	6.1		
0.01	407	1.5	424	−9.4	436	6.7			407	0.9	424	−2.9	436	5.3		
						10 mM PIPES/100 mM NaCl at pH 7.0										
0.25	400	0.2	419	−2.6	436	2.0			397	1.3	418	−5.3	440	4.1		
0.05	401	0.5	423	−6.3	436	4.6			397	1.3	422	−6.8	438	5.6		
0.01	407	1.5	425	−10.1	436	7.0			407	1.4	425	−7.9	437	7.0		
						No Buffer/10 mM NaCl at pH 7.0										
0.25	402	0.6	422	−4.8	434	4.5			402	0.4	422	−4.8	434	4.4		
0.05	403	0.6	422	−3.8	435	3.7		0.5	406	1.2	423	−8.9	435	6.8		0.9
0.01	405	0.5	424	−3.7	436	3.2		0.7	407	0.8	424	−4.4	436	3.7		1.2
						10 mM MES/10 mM NaCl at pH 6.0										
0.25	404	0.5	421	−2.1	435	2.3	457	0.7	404	0.4	421	−2.4	434	2.3	457	0.7
0.05	408	0.3	421	−0.3	434	1.5	451	2.2	409	0.3	422	−0.1		1.6	453	2.6
0.01							450	2.8							450	3.2
						10 mM MES/100 mM NaCl at pH 6.0										
0.25	403	0.3	420	−2.4	435	2.0			396	0.4	419	−3.3	437	2.2		
0.05	406	0.7	423	−5.0	435	4.1			397	1.1	422	−6.0	436	5.3		
0.01	407	1.1	424	−6.2	436	5.0			407	1.2	424	−6.9	436	5.4		
						No Buffer/10 mM NaCl at pH 6.0										
0.25	403	0.3	421	−1.8	436	2.0	458	0.8	405	0.4	422	−2.4	434	2.3	458	0.7
0.05	406	0.3	421	−0.4	435	1.5	451	2.0	409	0.4	422	−0.3		1.5	451	2.6
0.01							450	2.7							450	3.1

a shoulder at ~410 nm appeared on the short wavelength side of the −exc band. The [Θ] values at $R = 0.05$ were larger (e.g. 2.5 times for the −exc band) than at $R = 0.25$. Also at $R = 0.05$ in 100 mM NaCl, λ_{-exc} was 423 nm, a relatively large shift from 419 nm, the λ_{-exc} value at $R = 0.25$ in 100 mM NaCl. The shape of the spectrum at $R = 0.05$ was the same as observed initially in 10 mM NaCl, but intensities were 20−45% smaller in 100 mM NaCl. The ellipticities of the $R = 0.05$ spectrum increased by ~10−20% over 1 day for the −exc and +exc bands and by more than 200% for the +s band; however, the −exc and +exc values were still lower than the corresponding 1 day spectrum in 10 mM NaCl. The shoulder on the −exc band appeared after 1 day in the $R = 0.05$ spectrum (Figure S4). At $R = 0.01$, the molar ellipticities of the three bands were larger than those at $R = 0.05$ by 50−130%. After 1 day, the +s and +exc bands remained the same, but the −exc band decreased in intensity by ~20%. There was no shoulder on the −exc band after 1 day.

The CD experiment was repeated in the absence of PIPES (pH 7.0, 10 mM NaCl) (Figure S5). Soon after sample preparation, at $R = 0.25$, the CD spectrum showed the +s, −exc, and +exc bands with [Θ] values of 6×10^4, -4.8×10^5, and 4.5×10^5, respectively. At $R = 0.05$, the molar ellipticities of these three bands were ~15% smaller, and a small degree of ellipticity was observed at 451 nm. No ellipticity had been seen in this region in the presence of PIPES. The intensity of this **bHP** band at 451 nm increased by ~80% over 1 day (Figure S5b). At a higher concentration of CT DNA ($R = 0.01$), the intensities of the +s, −exc, and +exc bands were slightly less than those at $R = 0.05$, and the 451 nm feature was 40% larger than at $R = 0.05$. After 1 day, the conservative region of the $R = 0.05$ spectrum had increased while the $R = 0.25$ and 0.01 spectra changed only slightly over time, making the $R = 0.05$ spectrum the most intense of the three R values.

pH 6.0. The CD spectra at pH 6.0 showed distinct differences from those at pH 7.0, especially at low R values. First at $R = 0.25$ (10 mM MES, 10 mM NaCl), the +s, −exc, and +exc bands were smaller at pH 6.0 than at pH 7.0 (Figure 7b). Also at pH 6.0, there was a distinct band in the region of absorbance of the **bHP** species, with $[\Theta]_{457} = 7 \times 10^4$. At $R = 0.05$, the −exc band was reduced by ~90% from that observed at $R = 0.25$. The **bHP** band increased to 2.2×10^5 and formed a broad, flat feature with the +exc band. At $R = 0.01$, the +s, −exc, and +exc bands were essentially reduced to baseline, and a sharper peak was present at 450 nm. Over a period of 1 day, the spectra remained relatively unchanged.

In the presence of 100 mM NaCl (Figure 7a), at $R = 0.25$, the exciton region of the spectrum was similar to the one in 10 mM NaCl at $R = 0.25$. However, in 100 mM NaCl there was no **bHP** band. After 1 day, a small shoulder appeared on the −exc band. At $R = 0.05$, the CD spectrum was still exciton-like with larger intensities than at $R = 0.25$ and with 3 nm red shifts of the +s and −exc bands. Even though the shoulder on the −exc band had not been observed at $R = 0.05$ at $t = 0$, it was observed after 1 day. At $R = 0.01$, the exciton signal was larger than at $R = 0.05$. A distinct **bHP** band was not evident in the spectra for any of the three R values.

In the absence of MES (pH 6.0, 10 mM NaCl), at $R = 0.25$, the −exc and +exc bands of the CD spectrum had slightly smaller intensities than those in 10 mM MES (Figure 7c). The **bHP** band (shifted to 458 nm) was 15% larger in the absence than in the presence of buffer. After 1 day, the +s, −exc, and +exc bands increased by 10−20%, and the **bHP** band decreased by 15%. At $R = 0.05$, the intensities of the −exc and +exc bands were much smaller than those at $R = 0.25$. However, a very broad **bHP** band at 451 nm increased to $[\Theta]_{bHP} = 2.0 \times 10^5$. After 1 day, the **bHP** band increased by 30%. At $R = 0.01$, the conservative part of the spectrum was no longer present. The **bHP** band was sharper than at $R = 0.05$, and the intensity was larger by ~40%. After 1 day, the **bHP** band increased an additional 15%.

The effect of NaCl concentration on the CD spectra was also investigated (7.5 μM TθOPP, $R = 0.05$, pH 6.0, no buffer, Figure 8). In this study, the TθOPP−DNA adduct was titrated over the [NaCl] range 10−1000 mM. The wide positive CD band at ~451 nm was a distinct band at 10 mM NaCl, but with

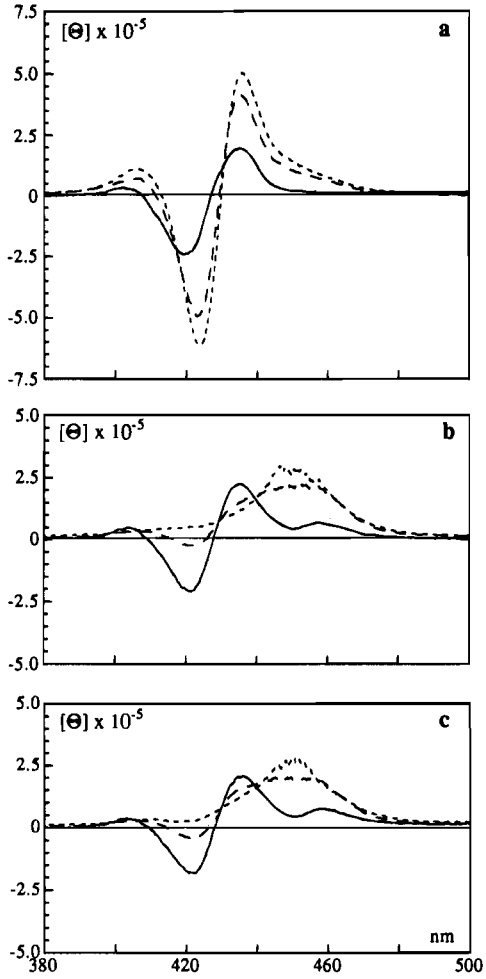

Figure 7. Circular dichroism spectrum of 7.5 μM TθOPP in the presence of CT DNA: (a) Solutions contained 10 mM MES buffer (pH 6.0), 100 mM NaCl; (b) solutions contained 10 mM MES buffer (pH 6.0), 10 mM NaCl; (c) solutions contained no buffer (pH 6.0), 10 mM NaCl; R = 0.25 (—); R = 0.05 (— —); R = 0.01 (- - -). Spectra acquired immediately after addition of DNA.

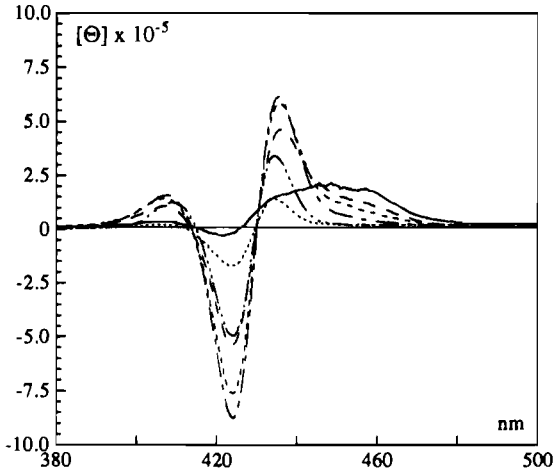

Figure 8. Circular dichroism spectrum of 7.5 μM TθOPP during NaCl titration of TθOP P/CT DNA adduct (R = 0.05): 10 mM NaCl (—); 50 mM (— — —); 100 mM (— — —); 200 mM (— - —); 500 mM (— --- —); 1000 mM (- - -); pH = 6.0; no buffer; T = 25 °C. The maximum ellipticity of conservative feature occurs in 200 mM NaCl.

increasing NaCl, the **bHP** band decreased in intensity and became a shoulder of the increasing +exc band. The exciton-type CD as well as the 408 nm band increased with increasing [NaCl] and exhibited maximal [Θ] values at 200 mM NaCl. In

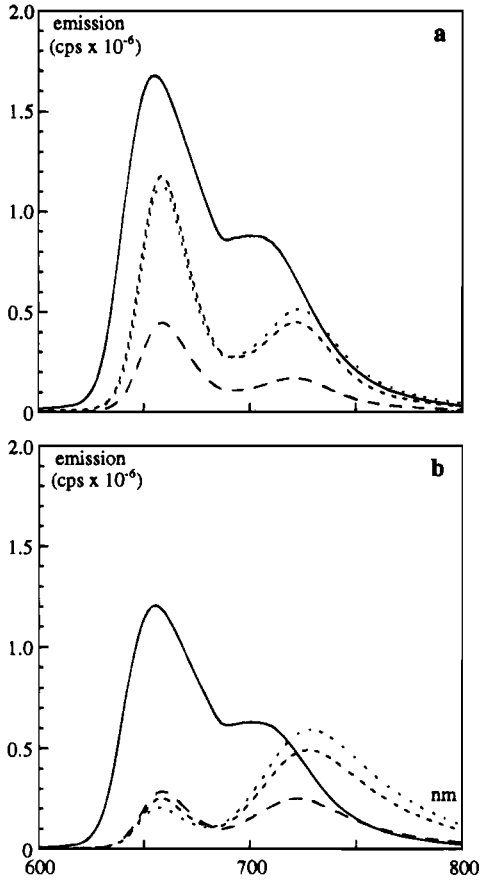

Figure 9. Fluorescence spectrum of 7.5 μM TθOPP at various porphyrin/DNA base pair ratios: (a) Solutions contained no buffer (pH 7.0), 10 mM NaCl; (b) solutions contained no buffer (pH 6.0), 10 mM NaCl; no DNA (—); R = 0.25 (— —); R = 0.05 (— — —); R = 0.01 (- - -). Spectra were acquired immediately after addition of DNA.

contrast, $[\Theta]_{bHP}$ decreased to 8×10^4 in 200 mM NaCl. The molar ellipticities of all bands decreased with increasing [NaCl] above 200 mM, such that at 1000 mM NaCl only a much smaller exciton signal remained with $[\Theta]_{-exc} = -1.8 \times 10^5$ and $[\Theta]_{+exc} = 1.5 \times 10^5$ and the +s and **bHP** bands were reduced to baseline.

Fluorescence Spectroscopy. The fluorescence spectrum of 1.0 μM TθOPP in water (pH 7.0) has two emission maxima at 653 nm [Q(0,0) band] and at ~700 nm [Q(0,1) band] (spectrum not shown, similar to Figure 9); the band assignment follows that used for other porphyrins.[35,36] The 653 nm band is the larger of the two, and the intensity ratio of these bands is ~2. Lowering the pH to 6.0 left the spectrum virtually unchanged, while, at pH 2.0, the protonated porphyrin exhibited only one emission band at ~720 nm.

In the presence of CT DNA (R = 0.25, pH 7.0, no buffer, 10 mM NaCl), the intensities of both bands decreased to ~25% of their values in the absence of DNA (Figure 9a). The 653 nm band was red-shifted to 659 nm, and the band at ~700 nm became better resolved from the 659 nm band with a shift to ~720 nm. Even at pH 7.7 (R = 0.25, no buffer, 10 mM NaCl) where TθOPP is fully unprotonated, the 720 nm band is present. Therefore, this band is associated with **bP**, not the **bHP** form. When R was decreased to 0.05 and to 0.01, the intensities increased with decreasing R, reaching values ~50% of those in the absence of DNA. For all three R values studied at pH

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7.0, the intensity of the 659 nm band is at least twice that of the ~720 nm band.

At pH 6.0 (no buffer, 10 mM NaCl), evidence was found for a protonated porphyrin (Figure 9b). At $R = 0.25$, the addition of DNA caused a large decrease in the 659 nm band, resulting in the band at ~720 nm being only 10% smaller than the 659 nm band. With decreasing R (0.05 and 0.01), the intensity of the 720 nm band increased while the 659 nm band underwent relatively smaller decreases, so that at $R = 0.01$ the 720 nm band is nearly three times as intense as the 659 nm band. The 720 nm band also underwent a slight red shift (725 nm at $R = 0.01$) with decreasing R. In a separate study, the pH of a TθOPP–DNA solution ($R = 0.05$, 10 mM NaCl) was lowered to 5.0, where the 659 nm band was no longer present and the longer wavelength band was shifted to 740 nm (Figure S6).

When the NaCl concentration was increased to 100 mM (pH 6.0), the 659 nm band remained more intense than the 720 nm band at $R = 0.25$, 0.05, and 0.01 (spectra not shown). Both bands increased in intensity with decreasing R in 100 mM NaCl.

In order to determine the effect of decreased self-stacking on its fluorescence spectrum, TθOPP fluorescence was monitored at $R = 0.25$, 0.05, and 0.01 (no buffer, 10 mM NaCl). The pH was maintained at 7.7 to prevent the effects of the formation of **bHP**. Fluorescence intensity increased with decreasing R, so that after 1 day, the 659 nm band at $R = 0.01$ was 2.5 times as large as at $R = 0.25$. The ratio of the intensities of the 659 nm and the 720 nm bands remained constant at all three R values. The same samples were examined by CD spectroscopy. At all three R values, large conservative features were observed, with no band at 451 nm. After 1 day, the relative intensities of the CD spectra at 424 nm were $R = 0.05 > 0.01 > 0.25$.

Discussion

Previous studies of the interaction of cationic porphyrins with nucleic acids have revealed three binding modes:[6-12] (1) *Intercalation* is characterized by a large bathochromic shift (at least 15 nm) and substantial hypochromicity (at least 30%) of the Soret band, by a single, *negative* induced CD band in the Soret region, by an increase in the solution reduced viscosity (SRV) of linear DNA, and by unwinding of closed circular supercoiled (CCS) DNA. Representative of this group is the TMpyP(4)–[poly(dGdC)]$_2$ system.[16] (2) *Outside binding without porphyrin stacking* displays a moderate bathochromic shift and hypochromicity (in some cases even hyperchromicity) of the Soret band, a single, *positive* induced CD band, no increase in SRV of linear DNA, and no unwinding of CCS DNA. An example of this type of binding can be found in the TMpyP(4)–[poly(dAdT)]$_2$ system.[16] (3) *Outside binding with porphyrin stacking* produces somewhat variable effects on the Soret band. Both a moderate bathochromic shift (9 nm) along with less than 30% hypochromicity, e.g. TMAP,[9] and a large (25–30 nm) shift with large (~60%) hypochromicity, e.g. *trans*-P(4),[37] have been observed. In the CD spectrum, conservative signals are indicative of outside binding with stacking and have been observed for TMAP,[9,15] *trans*-P(4),[37,38] and CuTMpy(4).[23,38] Partial intercalation, a fourth mode of binding, has not been well characterized by these optical spectroscopic methods.[13,14]

Below, we evaluate the binding mode of TθOPP to CT DNA in the context of these studies of other porphyrins. However, in light of the tendency of TθOPP to self-aggregate, it is important first to investigate the behavior of the porphyrin alone in aqueous solution.

Characteristics of Free TθOPP (No DNA). Many cationic water-soluble porphyrins undergo self-stacking in water. Self-stacking is usually accompanied by hypochromicity of the Soret band, broadening of the Soret band, and/or a shift in λ_{So}.[36,37,39,40] Large hypochromicities have been observed in the absence of DNA at high salt concentrations for two cationic porphyrins which are believed to self-aggregate: 55% for TMAP in 3.0 M NaCl[36] and 53% for *trans*-P(4) in 0.8 M NaCl.[37]

The dependence of the UV–vis spectrum of TθOPP on the concentration of NaCl or PIPES indicated that these species induced substantial self-stacking (Figure 2). The addition of NaCl caused a decrease in ϵ_{So} of TθOPP (65% hypochromicity at 200 mM NaCl), which was accompanied by a red shift of the shoulder at ~395 nm. For 7.3 μM TθOPP, the value of A_{So}/A_{sh} was ~5.5 in water and ~1.3 in 200 mM NaCl, with the lower ratio indicating a higher degree of self-stacking. Although the change in the spectrum was regular, no clear isosbestic point was observed, indicating that the self-stacking is not a simple monomer–dimer equilibrium. Beer's law spectroscopic studies of TθOPP self-aggregation at low porphyrin concentrations (<1 μM) were thwarted due to strong adsorption of the porphyrin onto the cuvette surface. However, in the determination of the molar absorptivity of TθOPP over the concentration range 2–7 μM (in 10 mM NaCl), the Beer's law plot showed a slight downward curvature at high concentration, deviating from linearity by only 3%, suggesting that the porphyrin is largely a single species at these concentrations. The addition of 10 mM PIPES to a 7.5 μM TθOPP solution caused 41% hypochromicity and a decrease in A_{So}/A_{sh} from 4.7 to 1.9. Both of these spectral changes are consistent with PIPES-induced self-stacking.

We believe the electron richness of the core of a water-soluble porphyrin to have a direct effect on its tendency to self-associate. One property which reflects electron richness is the pK_a of the protonated form of the porphyrin, and it has been proposed that aggregation increases with increasing pK_a.[41] In the absence of DNA, the pK_a of the protonated form of TθOPP (**HP**) is relatively high for a cationic porphyrin: 4.6 (1.8 μM TθOPP, 10 mM NaCl),[21] with slight variations caused by changes in porphyrin and salt concentrations. **HP** has a λ_{So} value of 445 nm, while the λ_{So} of **P** is 418 nm (Figure 1). The ability to distinguish these two species provides an additional spectroscopic handle with which to examine porphyrin–DNA interactions.

TθOPP–DNA Binding. We have previously reported several spectroscopic changes which occur upon formation of the **bP** species.[21,22] First, the Soret band undergoes a small bathochromic shift, usually accompanied by hypochromicity of the Soret band. Second, CD spectra are induced, the shape of which are dependent on the mode of TθOPP–DNA binding. Third, under appropriate conditions (low pH and/or low salt) bands at ~451 nm are observed in the UV–vis and CD spectra which are attributed to the **bHP** species. Finally, the 700 nm band in the fluorescence spectrum shifts to ~720 nm.

The spectroscopic changes described above can be analyzed in terms of several different DNA binding modes. To simplify our discussions, we suggest the following bound forms (Figure 10): an extensively stacked form without any protonation; a less stacked form still without protonation; a protonated, unstacked

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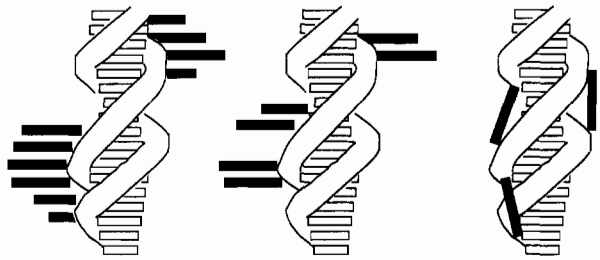

Figure 10. Representations of various modes of TθOPP–CT DNA binding. Shaded rectangles represent TθOPP bound to DNA.

form. These forms coexist under different conditions. The forms present at high and low R values are discussed, followed by a comparison with other self-stacking, cationic porphyrins which bind to DNA.

TθOPP–DNA Binding at High R. At $R = 0.25$ (pH 7.0, 10 mM PIPES, 10 mM NaCl), TθOPP binds on the outside of CT DNA as the **bP** species, as indicated by the small red shift of the Soret from 418 to 424 nm. In addition, the observed conservative CD pattern is consistent with an outside binding mode with self-stacking.[6,7] Our previous studies have shown no increase in DNA solution viscosity upon addition of TθOPP, suggesting that TθOPP does not intercalate.[21]

As discussed above, an increase in NaCl concentration caused increased stacking of TθOPP itself. In the presence of DNA, high salt concentration can weaken the porphyrin–DNA binding, which would result in a decrease in the induced ellipticity due to dissociation of the porphyrin from the DNA. On the other hand, high salt can affect the bound form by promoting stacking or altering the geometry of the stacked form; either effect could cause larger or smaller exciton coupling between porphyrin molecules. For the DNA-bound TθOPP ($R = 0.25$), an increase in [NaCl] from 10 to 100 mM caused increased porphyrin stacking, as indicated by the decrease in A_{So}/A_{sh} from 1.2 at 10 mM NaCl to 1.1 at 100 mM NaCl (pH 7.0, 10 mM PIPES). After 1 day, the CD intensities were much greater in 100 mM NaCl (Figure S4) than in 10 mM NaCl, and the ~410 nm shoulder appeared in the $R = 0.25$ and $R = 0.05$ spectra. Interestingly, this shoulder correlates to the ~410 nm shoulder in the absorbance spectra, which is sensitive to stacking. These results are consistent with a redistribution of TθOPP–DNA binding, which may involve decreased binding and/or altered stacking, with increased salt concentration.

The 400–435 nm region of the visible spectrum (Figure 4a) at pH 6.0 (10 mM MES, 10 mM NaCl) is similar to that at pH 7.0 (Figure 3a), suggesting a similar stacked TθOPP–DNA form at $R = 0.25$. However, at pH 6.0 an additional band at 451 nm was observed; the presence of this band indicates some protonation of the porphyrin at pH 6.0. The fluorescence spectra also indicate protonation at pH 6.0, as shown by the large intensity of the 720 nm band relative to the 659 nm band (Figure 9b). In the CD spectrum, an additional broad band at 457 nm, which is attributed to the **bHP** species, is also apparent at pH 6.0 (Figure 7). The same CD spectrum shows a conservative band with intensities ~50% of those at pH 7.0 (Figure S5). These findings for TθOPP at $R = 0.25$ in 10 mM NaCl suggest the formation of a **bP** adduct at pH 6.0 similar to that at pH 7.0; this form presumably is stacked although a small amount of **bHP** is present.

Having investigated the effect of high salt at high pH (7.0) and the effect of low pH in low salt (10 mM), we extended our studies to high salt (100 mM) at low pH (6.0) in order to understand more fully the salt dependence of the protonation process. In 100 mM NaCl at $R = 0.25$, the visible spectrum at pH 6.0 (Figure 4b) is similar to that at pH 7.0 (Figure 3b), suggesting the formation of a similar stacked TθOPP–DNA form. However, the lack of a distinct band at 451 nm in the pH 6.0 spectrum indicates no observed protonation at $R = 0.25$ in 100 mM NaCl, whereas in 10 mM NaCl a small degree of protonation was observed. At the higher salt concentration, there was no CD signal in the 451 nm region at pH 6.0 (Figure 7a). The intensity of the conservative feature in the CD spectrum in 100 mM was essentially the same as that in 10 mM NaCl (Figure 7b). Both the visible and the CD spectra suggest that increased [NaCl] caused decreased protonation as a result of extensive stacking of TθOPP.

TθOPP–DNA Binding at Low R. The form of the porphyrin–DNA complex is sensitive to the ratio of the two species. Overall, decreasing the porphyrin/DNA ratio increased the degree of protonation of the bound TθOPP. For $R = 0.05$ and 0.01 at pH 7.0 (10 mM NaCl, 10 mM PIPES), a small band at 451 nm in the visible spectrum was observed, indicating some protonation that was not present at $R = 0.25$ (Figure 3a). The intensity of this **bHP** band increased with decreasing R. The A_{So}/A_{sh} ratio increased from 1.2 at $R = 0.25$ to 1.4 at $R = 0.01$, suggesting partial unstacking. Also consistent with partial unstacking is the increase in ϵ_{So} and the >2-fold increase in fluorescence intensity from $R = 0.25$ to $R = 0.01$. If only the highly stacked form were present, then one would have expected the exciton-type CD signal to have become weaker as unstacking occurred. However, the conservative CD feature for $R = 0.05$ was *larger* by a factor of ~2 than that at $R = 0.25$ (Figure 6). This finding suggests that there is a moderately stacked form with a stronger conservative CD signal; this form is apparently quite stable at $R = 0.05$. Even at $R = 0.01$, the $[\Theta]$ values were still sizable, suggesting that even in a large excess of DNA, unstacking of bound TθOPP is still not complete, i.e., the bound TθOPP is partially stacked. Since similar results were observed at pH 7.7 (where no **bHP** is present), this unstacking is a result of greater distribution along the DNA backbone and not necessarily a result of TθOPP protonation.

In 100 mM NaCl, the spectra of TθOPP in the presence of DNA showed significant differences from those in 10 mM NaCl. At pH 7.0, no band for **bHP** was observed in either the UV–vis or CD spectra for R values as low as 0.01 (Figures 3 and S4), suggesting that protonation of TθOPP was inhibited in high salt. The intensities of the conservative CD signals at $R = 0.25$ had been smaller in 100 mM than in 10 mM salt, but at $R = 0.05$ and 0.01, the intensities were similar. The relatively large shift in λ_{-exc} from 423 nm at $R = 0.25$ to 425 nm at $R = 0.05$ suggests some change in the stacked form of TθOPP (Figure S4).

At pH 7.0 without PIPES (10 mM NaCl), the ϵ_{bHP} values were significantly larger at $R = 0.05$ and 0.01 than those observed with PIPES (Figures 3a and S2). The greater extent of protonation in the absence of PIPES is also supported by the presence of a shoulder at 451 nm in the CD spectra at $R = 0.05$ and 0.01 (Figure S5). The results indicate that the presence of PIPES disfavors formation of **bHP**. In addition, the A_{So}/A_{sh} values were slightly larger in the absence of PIPES, suggesting that PIPES promotes porphyrin stacking; this stacking may inhibit protonation. Thus, the visible and CD results suggest that PIPES affects the formation of the TθOPP–DNA complex; the negative groups of the PIPES in the dianion form presumably interact with the positive charges of the TθOPP tentacles on the opposite side of the porphyrin molecule from the tentacles which interact with the DNA duplex.

At pH 6.0 (10 mM MES, 10 mM NaCl), DNA solutions of TθOPP at $R = 0.05$ displayed an intense band in the visible spectrum at 451 nm and a positive CD band also at 451 nm (Figures 4a and 7b). These bands increased at $R = 0.01$, indicating an increase in the relative concentration of **bHP**. Increased protonation was also evident in the fluorescence spectra (Figure 9b). Furthermore, both pH 6.0 ($R = 0.05$ and 0.01) and pH 2 (no DNA) solutions were green, another indication of protonation. At $R = 0.01$, the conservative signal in the CD spectrum was reduced to baseline and only a positive band at 451 nm remained. This type of spectrum is consistent with an outside binding mode for the protonated porphyrin with no self-stacking.

At pH 6.0, all spectroscopic data showed a significantly lower extent of protonation in 100 mM than in 10 mM NaCl (small ϵ_{bHP} values (Figure 4b), large exciton CD signals (Figure 7a), and the near absence of the **bHP** CD band). The existence of the conservative CD bands indicates that some stacked, DNA-bound TθOPP is present at this high salt concentration. It is reasonable to assume that the greater stacking, which is facilitated by the higher salt, disfavors protonation.

Salt Effect. Our observations on the effect of increasing NaCl concentration on DNA-bound TθOPP can lead to several conclusions. First, the deprotonation of **bHP** caused by increased [NaCl] is indicated by (1) a decrease in ϵ_{bHP}, (2) an increase in ϵ_{So}, and (3) the disappearance of the 451 nm positive CD signal. Second, more extensive porphyrin stacking by increased [NaCl] is suggested by the induction of an exciton signal. Third, the shoulder at ~410 nm in the CD spectra is present only under conditions of extensive stacking, i.e., 100 mM NaCl, high R, and after 1 day. This shoulder is presumably related to a stacked form of TθOPP, although its exact nature is unclear. An expected effect of increased salt concentration is a decrease in the binding constant of porphyrin to DNA.[42,43] However, a NaCl titration of the TθOPP−CT DNA adduct (pH 6.0, $R = 0.05$, no buffer) showed substantial binding up to 200 mM NaCl, as indicated by a strong exciton CD signal (Figure 8). At higher NaCl concentrations (200−1000 mM), the exciton signal as well as the **bHP** band gradually decreased with increasing [NaCl], suggesting decreased binding of the porphyrin to DNA.

Time Dependence of Spectra. The absorbance and CD spectra of TθOPP showed time dependence under certain conditions. Typically, in low salt, there was a gradual increase in the degree of protonation over time, as indicated by an increase in the intensities of the **bHP** bands in the absorbance and CD spectra. At pH 6.0 in low salt, the intensity of the **bHP** band in the visible spectrum increased as much as 80% during the first hour after DNA addition. In high salt at both pH 6.0 and 7.0, no significant changes in the **bHP** band intensity were observed over 1 day. In general, it appears that an equilibrium is reached for proton uptake by the porphyrin core within the first few hours after sample preparation. The exact rate of proton uptake is dependent on specific solution conditions, especially those that affect self-stacking of TθOPP, such as R value and salt concentration.

Comparison to Other Self-Stacking Porphyrins. In studies of other cationic porphyrins, both TMAP and *trans*-P(4) exhibit conservative CD spectra in the presence of CT DNA and both have been characterized as outside binders with self-stacking.[9,37,44] In the presence of CT DNA, TθOPP also exhibits a strong conservative CD spectrum at appropriate pH and salt conditions, indicating that this porphyrin also undergoes self-stacking. In addition to the conservative feature, a small positive band is present at 400−410 nm in the CD spectrum of TθOPP. A metalloporphyrin, CuTMpyP(4), which has also been found to self-stack along the surface of [poly(dAdT)]$_2$ ($R = 0.10$, pH 7.8), has a strikingly similar CD spectrum to that of TθOPP in the presence of CT DNA, including the small positive band at ~410 nm.[23] These results suggest that the orientations of the two porphyrins are similar; both porphyrins are outside-bound. Interestingly, CuTMpyP(4) is an intercalator with [poly(dGdC)]$_2$, with no self-stacking indicated by the CD spectrum.[23]

The binding mode of self-stacking porphyrins is often affected by changes in R value. For TMAP with CT DNA, other investigators have shown that the negative band of the conservative CD signal decreased with decreasing R, such that, at $R <$ 0.1, only a positive band remained.[9,15] It was suggested that, at low R, the conservative CD spectrum is lost because the TMAP molecules are no longer highly stacked along the DNA surface; at low R the binding mode is either "face on" or "edge on" binding of isolated molecules.[9,15] For *trans*-P(4), at low R (0.044) with CT DNA, only a 9 nm red shift of the Soret with 42% hypochromicity was observed, as well as a single negative CD band with very small ellipticity.[37] These hypochromicity and CD features are characteristic of intercalation, although at high R (1.74) *trans*-P(4) underwent outside binding with self-stacking, as evidenced by a conservative CD spectrum.[37] These results suggest that DNA in large excess is able to convert the stacked *trans*-P(4) at high R completely to the intercalated form at low R. TθOPP also undergoes a change in binding mode with decreasing R (Figure 7). At $R = 0.25$ (pH 6.0, low salt), the conservative CD feature suggests outside binding with stacking, but at higher DNA concentrations ($R = 0.01$), only a single positive CD band is present, suggesting outside binding without stacking. In addition, the absorbance spectra at pH 7.0 suggest some unstacking of TθOPP at low R, as shown by increases in A_{So}/A_{sh} and by decreases in hypochromicity. However, these changes could be due exclusively to a less stacked form of TθOPP.

The salt concentration influences both the extent of porphyrin stacking along DNA and the mode of porphyrin−DNA binding. The *trans*-P(4)−[poly(dAdT)]$_2$ system had a single positive CD band and only 10% hypochromicity at low salt (suggesting outside binding without stacking), but at high salt, H increased to 51% and an exciton CD signal with large ellipticity was induced, consistent with outside binding with stacking.[37] The *trans*-P(4)−[poly(dGdC)]$_2$ system showed a small negative CD band in 10 mM NaCl, consistent with intercalative binding, but an exciton pattern was found in 87 mM NaCl.[37] Similarly in this study of TθOPP, a change in binding mode from simple outside binding to outside binding with stacking was observed at pH 6.0 ($R = 0.01$) upon increasing [NaCl] from 10 mM to 100 mM. These changes suggest that stacking is favored by high salt.

Next to protonated TθOPP, protonated TMAP has the highest pK_a (3.6 in 0.2 M $NaNO_3$)[31] of the cationic porphyrins for which DNA binding studies have been reported. This pK_a is evidently too low to support protonation of the bound form of the porphyrin.

In our studies, the degree of protonation of TθOPP often changed over time. Time-dependent interactions between *trans*-(P4) and DNAs have also been reported.[37] Both the absorbance

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and CD signals changed with time when *trans*-(P4) interacted with $[poly(dGdC)]_2$, $[poly(dAdT)]_2$, or CT DNA. For TMAP, no such effects have been reported. The time-dependent phenomena most probably reflect the tendency of the porphyrin to self-stack, particularly along the outside of DNA.

Conclusions

TθOPP is primarily an outside binder with CT DNA, as shown by previous viscometric studies,[21] as well as by UV−vis, CD, and fluorescence spectroscopy. Outside binding is seen at all NaCl concentrations (10−1000 mM NaCl) and R values (0.25−0.01) studied. Due to the greater electron richness of its porphine core compared to previously studied DNA-binding porphyrins, TθOPP undergoes a substantial degree of self-stacking along the DNA surface even at relatively low NaCl concentrations (10 mM NaCl). There are at least two outside-bound, self-stacked forms of TθOPP: one highly stacked with a less intense conservative CD feature and a less stacked form with a more intense conservative CD feature. Intermediate stacked forms probably exist. The less stacked form seems to be most stable at $R = 0.05$. At $R = 0.01$, the less stacked form is metastable and partially unstacks with time. This unstacking may allow the tentacles to reach out and interact with a more extended region of DNA, i.e., have a bigger footprint than the more stacked form.

TθOPP is the first porphyrin reported to form a protonated porphyrin−DNA adduct.[22] With its positive charges located at the end of the tentacles and relatively far from the central core, protonated TθOPP has the highest pK_a value of any cationic porphyrin reported to date for which DNA binding studies have been performed; protonation is therefore possible at pH values at which duplex B-form DNA can be studied. Protonation is favored at low R. Furthermore, the degree of protonation of the porphyrin is reduced under conditions which promote the stacking of TθOPP, i.e., in high salt or with PIPES buffer. Thus, under conditions where stacking along the DNA is reduced, the extent of porphyrin protonation becomes high. At pH 6.0 in 10 mM NaCl, the bound tentacle porphyrin is largely both protonated and unstacked. Under many conditions, relatively slow changes in the bound forms of TθOPP are observed. For this porphyrin, the amount of protonation is time dependent because protonation is a function of stacking and the stacking/unstacking process can occur over many hours. Since the total contents of the solution remain unchanged, the increase of protonation with time suggests that it is the unstacked porphyrin which becomes protonated. The protonation step itself should be very fast for an unstacked, bound species, just as it is fast for the unbound porphyrin.

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Supplementary Material Available: Figures showing UV−vis absorbance and fluorescence spectra of a pH titration of the TθOPP−DNA adduct, UV−vis absorbance spectra of the TθOPP−DNA adduct in the absence of buffer, and circular dichroism spectra of the TθOPP−DNA adduct in high salt concentration and in the absence of buffer (6 pages). Ordering information is given on any current masthead page.