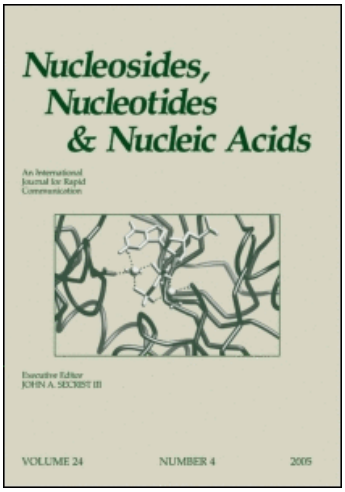

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Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 26 February 2001

To cite this Article Bichenkova, Elena V. , Frau, Silvana , Fedorova, Olga S. and Douglas, Kenneth T.(2001) 'BINDING OF A DESMETALLO-PORPHYRIN CONJUGATE OF HOECHST 33258 TO DNA. III. STRONG BINDING TO SINGLE-STRAND OLIGONUCLEOTIDES', Nucleosides, Nucleotides and Nucleic Acids, 20: 1, 157 — 168
To link to this Article: DOI: 10.1081/NCN-100001444
URL: http://dx.doi.org/10.1081/NCN-100001444

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(1&2), 157–168 (2001)

BINDING OF A DESMETALLO-PORPHYRIN CONJUGATE OF HOECHST 33258 TO DNA. III. STRONG BINDING TO SINGLE-STRAND OLIGONUCLEOTIDES

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ABSTRACT

The binding of the conjugate of Hoechst 33258 with 5,10,15,20-tetrakis (1-methyl-4-pyridyl)-21H,23H-porphyrin (PORHOE) to single-strand DNA has been detected by UV–vis spectrophotometry and ^{1}H-NMR. The red-shift of porphyrin Soret band with strong hypochromicity indicates that the porphyrin moiety dominates in the interaction of the PORHOE with ssDNA. The affinity constants of PORHOE for d(GCATACAATTCG) or d(CGAATTGTATGC) were determined to be $>10^5\ M^{-1}$, with strong cooperativity.

Key Words: Single strand DNA; Ligand; NMR; Binding constant.

INTRODUCTION

Porphyrin binding to double-stranded (ds) DNA has been extensively studied and Feng et al. determined binding constants for calf thymus DNA of 0.5–0.9 $\times$ $10^5\ M^{-1}$ (1) based on their own as well as literature binding data for tetra-*N*-methylpyridiniumporphine (H2TMPyP-4) for the initial outside binding of the first

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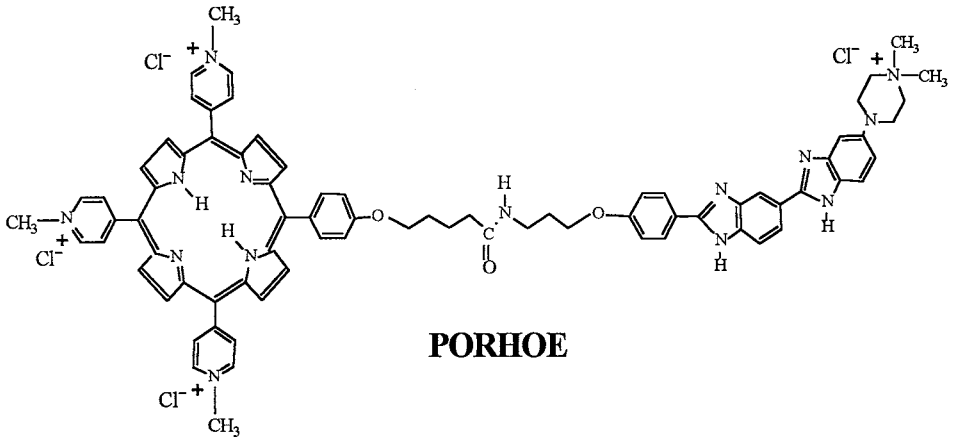
porphyrin molecule prior to interaction caused by subsequent ligand site occupancy. Metal-free H2TMPyP-4 has been established by a wide range of techniques to intercalate selectively at GC-rich regions of duplex DNA with reported binding constants of $\sim 7 \times 10^5\ M^{-1}$ for the poly d(G–C) · poly d(G–C) duplex (2–4). Some cationic porphyrin ligands interact with dsDNA nonintercalatively, that is, hemi-intercalation and outside random binding (5–9).

There are as yet few ligands reported that bind strongly to single-stranded (ss) DNA without subsequent chemical reaction with the nucleic acid. Many reagents that exhibit apparent single-strand binding do so by interaction with locally double-stranded regions or other special structural features such as hairpins. Thus, a cationic porphyrin has been suggested to form transient, intercalation-like complexes with single-stranded DNA *en route* to duplex formation (10). Single-stranded nucleic acids have also been used as templates to assemble stacks of cationic porphyrins(11).

Recently, high affinity binding has been detected for ssDNA with a tetraphenyl-porphyrin conjugated to a peptide sequence designed from a known single-strand DNA binding sequence (Lys–Trp–Lys) (12). In the accompanying studies (Parts 1 and 2) the interaction of a 12-mer oligonucleotide duplex with porphyrin–HOE 33258 (PORHOE) was investigated, where PORHOE is the conjugate of Hoechst 33258 (HOE) with 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21H,23H-porphyrin, tetra-*p*-tosylate salt (POR).

Unusual behavior for the porphyrin moiety of PORHOE was detected in DNA melting profiles monitored at 430 nm (corresponding to λ_{max} of unbound porphyrin) as the probe wavelength for the thermal stability of the complex. Even at 100°C, after complete duplex dissociation, the porphyrin moiety of PORHOE remained associated with the DNA in some way. It appeared possible that the porphyrin moiety could bind strongly to a single-strand oligo partner. We now provide direct observations of high affinity binding of PORHOE to single-strand DNA 12-mer oligonucleotides in comparison with its components (HOE and POR).



PORHOE

Scheme 1.

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MATERIALS AND METHODS

Reagents

The conjugate PORHOE was synthesized and characterized as previously reported (13,14). HOE 33258 and 5, 10, 15, 20-tetrakis (1-methyl-4-pyridyl)- 21H, 23H-porphyrin, tetra-*p*-tosylate (POR) were from Aldrich . The 12-mer oligonucleotides, CGAATTGTATGC and GCATACAATTCG were purchased from the Oswell DNA unit (University of Southampton).

UV–Vis Spectrophotometry

UV–vis spectra were measured on a Cary–Varian 1E with operating system/2 (version 3) and CARY 1 software. Experiments on comparative binding of PORHOE, HOE and POR with ss d(CGAATTGTATGC) were performed both in H_2O and in buffer A (10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, 100 mM NaCl, 0.1 mM EDTA). UV–vis spectra of ligands, both free and bound to d(CGAATTGTATGC), were recorded at 20°C in the absence and presence of 1 M NaCl. In all cases equal concentrations (2 μM) of ssDNA and ligand were used.

To determine the binding constants of PORHOE for d(GCATACAATTCG) and d(CGAATTGTATGC) oligonucleotides, titrations of PORHOE by the respective oligonucleotide were monitored at the λ_{max} of the porphyrin moiety (432 nm). The appropriate oligonucleotide was added stepwise to PORHOE (3 ml of 1.65 μM in buffer A). In the case of d(GCATACAATTCG) small aliquots (10 each of 2 μl) and then two aliquots (each 20 μl) of oligonucleotide stock (247.5 μM) were added sequentially to give a final concentration of 4.95 μM in the cuvette. In the case of d(CGAATTGTATGC), the titration protocol of PORHOE (1.65 μM, 3 ml) by oligonucleotide stock solution (247.5 μM) was 10×1 μl then 5×2 μl followed by 5×20 μl, giving a final oligonucleotide concentration of 9.9 μM. After each addition sample was allowed to equilibrate thermally for 10 min.

NMR Experiments

NMR data were collected on a Varian Unity 400 NMR spectrometer (400 MHz) operating at 400.130 MHz and equipped with a Sun Sparc I host computer running VNMR system software version 4.3. A dual (inverse) $^1H(X)$ probe-head was used for all proton observations. The NMR titration of d(CGAATTGTATGC) by PORHOE was performed at 25°C by recording the spectra of oligonucleotide with increasing mole fraction of PORHOE. The required aliquots of PORHOE stock solution (2 mM) were added stepwise to the NMR tube containing oligonucleotide (0.55 mM) in buffer A prepared in 99.96% D_2O. Data were collected into 16K data points over a spectral width of 5000 Hz, giving a final resolution of 0.31 Hz/point. For each spectrum 128 transients were acquired with 2.5 s recycle delay. NMR

data sets were referenced internally to the singlet methyl resonance of sodium 3-(trimethylsilyl-2,2,3,3, H_4)-1-propionate at 0 ppm.

RESULTS

UV–vis spectrophotometric data were used to study binding between single-stranded d(CGAATTGTATGC) and PORHOE, both in H_2O (Fig. 1A) and in buffer A (Fig. 2A), in comparison with binding of the separate moieties HOE and POR (see B and C, respectively, in Fig. 1 and Fig. 2). In all cases equal concentrations (2 μM) of ligand and ssDNA were used. Table 1 summarizes the effect of ssDNA binding (1:1) on λ_{max} and $\varepsilon_{\lambda_{max}}$ for PORHOE, POR, and HOE in the absence and presence of NaCl (1 *M* final concentration). Similar data (not presented) were obtained for single-stranded d(GCATACAATTCG).

From Figure 1B and Table 1, addition of ss d(CGAATTGTATGC) to HOE in water at pH 5.5 caused λ_{max} to shift from 341 to 356 nm, accompanied by a strong hypochromic effect (~50%), indicating complex formation between ligand and ssDNA. However, the dye was completely released from ssDNA–HOE complex by addition of 1 *M* NaCl. (HOE absorbance intensity did not completely recover in 1 *M* NaCl due to the high ionic strength; shown in separate experiment.) At pH 5.5, the benzimidazole rings of HOE are likely to be partially protonated (15) and thus positively charged. Thus, binding of HOE to ssDNA in water is a result of nonspecific electrostatic interactions. In contrast, HOE does not bind to ssDNA in buffer (Fig. 2B and Table 1), perhaps because the higher pH (7.0) deprotonates the benzimidazole rings, and/or because of the presence of counter cations (Na^+), which may compete with HOE for weak binding to negatively charged ssDNA.

Free porphyrin also binds to ssDNA in water (Fig. 1C and Table 1), resulting in a λ_{max} shift from 422 to 438 nm and a strong hypochromic effect. The addition of counter cations (Na^+) caused complete release of the porphyrin from the ssDNA–ligand complex with λ_{max} shifting back to 423 nm with partial recovery of intensity. (The observed decrease of porphyrin intensity when the added NaCl concentration was 1 *M* was shown in separate experiments to be attributable to the high ionic strength.) In buffer (Fig. 2C) there is an equilibrium between the unbound and bound states of porphyrin: the λ_{max} measured (430 nm) represents an intermediate value between λ_{max} for unbound (422 nm) and bound (438 nm) states. This equilibrium can be shifted towards the unbound state by addition of salt (1 *M* NaCl), indicating strong nonspecific binding between positively charged porphyrin and negatively charged ssDNA.

The behavior HOE and POR components of PORHOE with ssDNA (Figs. 1A and 2B, Tab. 1) is similar to that of free Hoechst and free porphyrin, respectively, both in water and in buffer, but with a less pronounced λ_{max} shift. The binding of PORHOE to ssDNA caused a red-shift of the porphyrin Soret band from 429 to 434 nm in water, and from 432 to 436 nm in buffer, accompanied by 36% and

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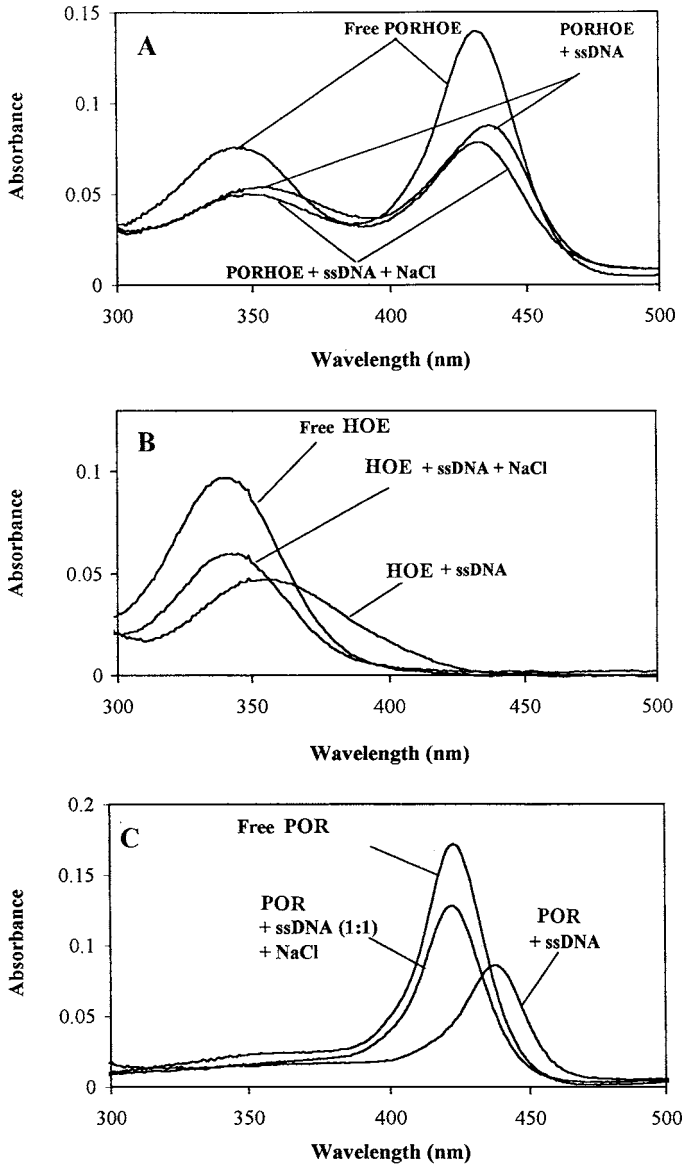

Figure 1. UV–vis spectra of ligands, both free and bound to d(CGAATTGTATGC), measured at 20°C in H_2O (pH 5.5) in the absence and presence of 1 *M* NaCl. Spectra are annotated with respective system compositions. In all cases equal concentrations (2 μM) of ssDNA and ligand were used: A) PORHOE; B) HOE; C) POR.

28% hypochromicities, respectively. The HOE moiety of PORHOE, similarly to free HOE under the same conditions, binds to ssDNA in water (red-shift of λ_{max} from 342 to 351 nm), but remains unbound in buffered conditions. PORHOE was released from the complex by addition of 1 *M* NaCl (λ_{max} recovered its original

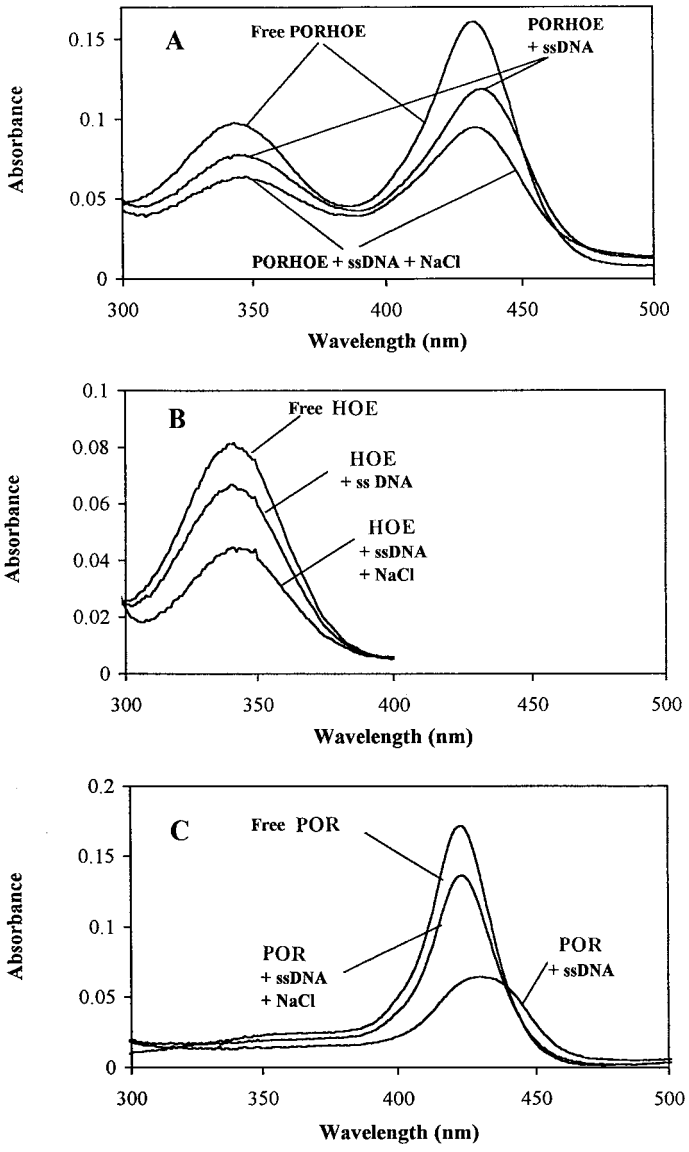

Figure 2. UV–vis spectra of various ligands, both free and bound to d(CGAATTGTATGC), measured at 20°C in 10 m*M* sodium phosphate buffer, pH 7.00, containing 100 m*M* NaCl and 0.1 m*M* EDTA. Spectra are annotated with respective system compositions. In all cases equal concentrations (2 μM) of ssDNA and ligand were used: A) PORHOE; B) HOE; C) POR.

value, although the intensity did not, due to the high ionic strength). Thus, the electrostatic interaction of the POR component with ssDNA is greater than for HOE, especially in the presence of counter-cations. Therefore, the POR moiety seems to serve as an "electrostatic anchor" in the interaction of PORHOE with ssDNA in buffered conditions.

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Table 1. UV–vis Spectrophotometric Study of Interactions Between PORHOE, POR, and HOE with Single-Stranded d(CGAATTGTATGC) at 25°C in pH 7.00 Buffer (10 mM NaH_2PO_4/Na_2HPO_4, 100 mM NaCl, 0.1 mM EDTA in H_2O). Data Are for an Equimolar Ratio of ssDNA (2 μM) and the Respective Ligand

	Water (pH 5.5)		Buffer A (pH 7.0)	
ssDNA–Ligand System	λ_{max} (nm)	ε_{max} (M^{-1} cm^{-1})	λ_{max} (nm)	ε_{max} (M^{-1}cm^{-1})
HOE (free)	341	40,100	341	38,450
HOE + ssDNA (1:1)	356	22,850	343.9	21,900
HOE + ssDNA + 1M NaCl	342.5	22,150	344	22,150
HOE + 1M NaCl	343.9	22,100		
POR (free)	421.5	64,000	422.4	70,450
POR + ssDNA (1:1)	438.1	43,100	430.2	32,000
POR + ssDNA + 1M NaCl	423.7	68,400	423.7	68,400
POR + 1M NaCl	423.2	75,000	423.2	75,000
PORHOE (free)	429	68,750	342	80,600
	342	37,500	344	48,450
PORHOE + ssDNA (1:1)	434	43,500	436	58,500
	351	27,000	345	38,500
PORHOE + ssDNA + 1M NaCl	430	39,000	433	47,000
	346	24,500	345	31,500
PORHOE + 1M NaCl	428	45,300	432.7	34,800
	342	27,750	344	19,000

The change of absorption at 432 nm (ΔA) as a result of binding of PORHOE to ssDNA (Fig. 3A) is described by Equation (1):

$$\Delta A = \frac{\Delta A_\infty}{L_0}[\text{complex}], \tag{1}$$

where ΔA_∞ is the change of absorption at [oligo] $\rightarrow \infty$, L_0 is the total concentration of PORHOE, and [complex] is the concentration of bound PORHOE. Titration data for PORHOE with increasing concentrations of d(CGAATTGTATGC) were analyzed in terms of a single binding site and two binding sites per oligo, by explicit solution of the equilibrium equations. In neither case was the fit judged to be good. Applying the McGhee–Von Hippel approach (16) gave a better fit when cooperativity was incorporated into the model, Equation (2). This requires that the approximations of this model, originally developed for ligand binding to DNA of infinite length, can also be used for the shorter oligonucleotides of this study.

$$\frac{v}{L} = K(1-nv)\left[\frac{(2w-1)(1-nv)+v-R}{2(w-1)(1-nv)}\right]^{n-1}\left[\frac{1-(n+1)v+R}{2(1-nv)}\right]^2 \tag{2}$$

In Equation (2), K is the apparent association constant of ligand L with an isolated binding site of oligonucleotide, n the neighbor exclusion value (the number of consecutive DNA nucleotides that become inaccessible on binding of one ligand

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molecule), v the ratio of moles of bound ligand (ligand–oligonucleotide complex) to total moles of nucleotides of oligonucleotide (N_0), L the concentration of free (unbound) ligand, and

$$R = \{[1 - (n+1)v]^2 + 4wv(1 - nv)\}^{1/2} \tag{3}$$

where w (the cooperativity parameter) equals the ratio of ligand binding constant with an isolated oligo site to that for a binding site close to the occupied site. Changes of absorption ΔA were measured experimentally and values of v and L calculated according to Equations (4) and (5):

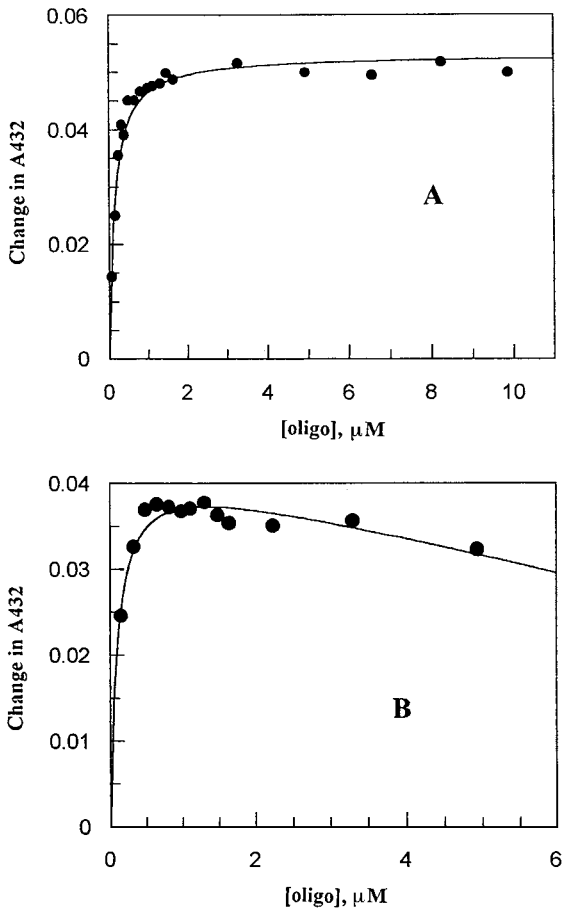

Figure 3. Binding isotherms for PORHOE. A). Titration of 1.65 μM PORHOE with increasing concentrations of d(CGAATTGTATGC) at 20°C, monitoring at 432 nm. Points are experimental: line is notional to assist visualization. B). Titration of 1.65 μM PORHOE with increasing concentrations of d(GCATACAATTCG) at 20°C. Points are experimental: line is notional to assist visualization.

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$$v = \frac{L_0}{N_0} \cdot \frac{\Delta A}{\Delta A_\infty}; \tag{4}$$

$$L = L_0 \cdot \frac{\Delta A_\infty - \Delta A}{\Delta A_\infty}. \tag{5}$$

The fitting procedure using these equations gave $K = (3.15 \pm 0.75) \times 10^5 M^{-1}$, $n = 2.07 \pm 0.11$ and $w = 10.00 \pm 3.15$, indicating that each PORHOE molecule occupies two nucleotide units in the oligonucleotide molecule and that the binding is strongly cooperative.

The binding of PORHOE to d(GCATACAATTCG) was even more complex with at least two classes of binding sites evident from the two regions as seen in Figure 3B. In the first of these regions ΔA increases with added oligo concentration; in the second, as [oligo] $\geq 1\ \mu M$, the ΔA decreases showing additional classes of weak binding site.

The dynamics of PORHOE binding to single-stranded DNA were also monitored by ^{1}H-NMR spectroscopy using titration of d(CGAATTGTATGC) by ligand. Figure 4 shows a series of 1D ^{1}H-NMR spectra (aromatic regions) of d(CGAATTGTATGC), both free and in the presence of increasing mole percentage PORHOE, recorded at 25°C in buffer A prepared in D_2O. The spectra are annotated with the percentage mole fraction of PORHOE relative to the initial concentration of oligonucleotide. The observed line broadening ($\Delta\nu_{1/2}$ in the range 2.0–4.2 Hz) and change in chemical shifts ($\Delta\delta$ goes from -0.007 to -0.014 ppm) for oligonucleotide protons resulting from stepwise addition of PORHOE clearly indicate the binding of ligand to the oligonucleotide single strand. It should be mentioned that no specificity in line broadening, connected with either nucleotide nature or nucleotide location within the strand, was observed, although line broadening was more pronounced for purine-derived protons than for pyrimidines, perhaps due to higher hydrophobic and/or stacking interactions between the the ligand and purine bases. These data again confirm the nonspecific character of the interaction between ssDNA and PORHOE, which may be slightly affected by the hydrophobic properties of particular heterocyclic bases.

DISCUSSION

The UV–vis spectrum of the PORHOE conjugate is strongly perturbed by binding to duplex d(CGAATTGTATGC)·d(GCATACAATTCG) (see Part I of this series, accompanying manuscript). The λ_{max} of the Hoechst moiety shifts from 340 to 350 nm, characteristic of the minor groove binding of Hoechst 33258 and analogs to duplex DNA, which has been confirmed and analyzed in great detail by high resolution structural techniques both in the crystal and solution (15,17–23). The Soret band of the porphyrin group shifts from 430 to 437 nm with a very strong

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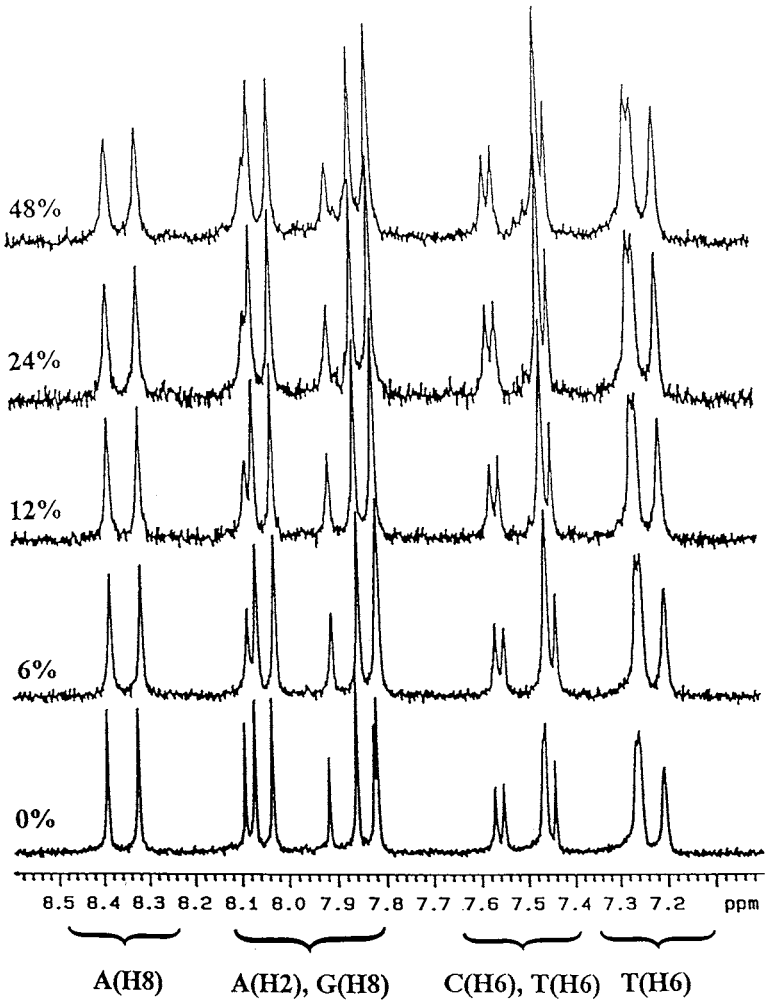

Figure 4. Aromatic regions of a series of 1D ^{1}H-NMR spectra (400 MHz) of single-stranded d(CGAATTGTATGC), both free and with increasing mole percentage of PORHOE, recorded at 25°C in 10 m*M* sodium phosphate buffer pD 7.00, containing 100 m*M* NaCl and 0.1 m*M* EDTA. The oligonucleotide concentration in the NMR tube was 0.55 m*M*. Spectra are annotated with percentage mole fractions of PORHOE relative to initial oligonucleotide concentration.

hypochromicity (approximately 3-fold), characteristic of *des*-metallocationic porphyrins binding to duplex DNA (24,25).

The binding of the tetraphenylporphyrin-peptide conjugate to single-strand oligonucleotides showed a >50% hypochromicity of the Soret band with little shift in λ_{max} (12). For PORHOE in the present study a hypochromic effect and λ_{max} shift was observed with single-stranded oligonucleotides for the Soret band of the porphyrin moiety, either at 20°C or 100°C, and affinity constants of greater than $>10^5 M^{-1}$ were determined at 20°C for the interaction with d(CGAATTGTATGC).

Since the λ_{max} value corresponding to the HOE component of PORHOE did not change, and the intensity of the absorption was only slightly affected, it is reasonable to conclude that binding of PORHOE to single-stranded DNA is dominated by the interaction of the porphyrin moiety.

This interaction must be a particularly favored structural arrangement as the binding strength is at least as strong as (and may even be greater than) that of an isolated porphyrin to duplex DNA, and the binding is maintained even at very elevated temperatures. The McGhee–Von Hippel analysis shows that the binding in buffered media at pH 7 of PORHOE to ssDNA can occur in such a way that the n value is 2, that is, only two nucleotides become inaccessible on binding of one ligand molecule. A physical model consistent with this is for the final equilibrium structure of the ssDNA–PORHOE complex to have its main interactions through the porphyrin group, as the length of the Hoechst region is closer to four or five nucleotide units.

For the strong, ss binding of *des*-metallo cationic porphyrins recently seen for porphyrins linked to ss-binding peptide (Lys−Trp−Lys) (12) it is not apparent how the dissociation constant of the DNA–ligand complex was estimated as $\leq$5 nM (12), as the concentrations used indicated essentially stoichiometric titration. Thus, a direct comparison of the strengths of interaction is not possible for this peptidic porphyrin and PORHOE. In water, as opposed to buffered media, the Hoechst region of PORHOE may be involved in ssDNA binding as there are potentially up to three additional cationic nitrogen sites in the Hoechst section. Because of the high flexibility of the side chains of lysine, the distribution of such positive sites, which could make essentially nonspecific electrostatic interactions with the phosphates on the ssDNA strand, can be modeled to be similar to those in the Lys−Trp−Lys peptide of the Porphyrin−Lys−Trp−Lys single-strand DNA probe recently reported (12). The binding constant of $3.15 \times 10^{-5} M^{-1}$, which we have determined for PORHOE binding to ssDNA, represents a lower limit for the analogos binding constant describing outside edge binding of a porphyrin to ds-DNA as the binding of PORHOE in buffer is reasonably approximated as mainly that of the porphyrin contribution. The estimate this approach gives must be regarded as a lower limit because the distribution of phosphate negative charges on dsDNA is fixed geometrically and hence the major difference in ligand structure on binding to target DNA, comparing ss and ds DNA, is the entropic sacrifice that the former DNA must make in achieving a complex with its phosphate sites presumably specifically distributed with respect to occlusion of porphyrin positive charges.

ACKNOWLEDGMENTS

We are grateful to the EU for the provision of a Marie-Curie Postdoctoral Fellowship (SF) and to the BBSRC for support (EVB), to Dr. Martin Baron for access to the 400 MHz instrument.

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Received March 8, 2000
Accepted September 29, 2000

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