DNA binding and intercalation by novel porphyrins: role of charge and substituents probed by DNase I footprinting and topoisomerase I unwinding

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Porphyrins carrying four charged sidechains, e.g., *meso*-tetrakis[4-*N*-methylpyridiniumyl]- and *meso*-tetrakis[4-*N*-(2-hydroxyethyl)pyridiniumyl]-porphyrin, bound and intercalated similarly into DNA as measured by helix stabilization and DNA unwinding studies in the presence of DNA topoisomerase I. Despite their different bulky sidechains, these complexes gave essentially identical DNase I footprinting patterns. In contrast, tetrasubstituted porphyrins carrying three phenyl rings and a single positively charged pyridiniumyl sidechain did not intercalate and exhibited little affinity for DNA. Thus, the presence of charged sidechains on the porphyrin rather than their identity appears to be critical for efficient DNA intercalation. The results are discussed in regard to current models for the porphyrin-DNA intercalation complex.

Porphyrin; DNase I footprinting; DNA unwinding; Helix stabilization; DNA intercalation

1. INTRODUCTION

DNA intercalators are usually small planar molecules containing fused aromatic rings [1]. However, certain porphyrins are also known to bind and intercalate into DNA [2]. The porphyrin ring system is larger and more complex than that of other known intercalators and poses some intriguing questions for DNA ligand interactions [3-6]. Most studies of porphyrin-DNA binding have focused on *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin, which carries four cationic substituents (compound **2a**, fig.1). A variety of approaches has shown that the presence and identity of the metal ion determines the DNA binding mode and DNA binding specificity of this porphyrin [3-6]. Mn^{3+}, Fe^{3+} and Zn^{2+} complexes bind solely by a non-intercalative mechanism to AT-rich regions of DNA, whereas the metal-free ligand and its Cu^{2+} and Ni^{2+} complexes bind intercalatively to GC-rich sequences and with less preference at TA sequences. The molecular basis underlying this sequence specific DNA intercalation is not known, particularly the role of the bulky noncoplanar porphyrin sidechains, which at first sight might be expected to inhibit intercalation. Similarly, the role of the positive charges on these side chains is not understood. We have approached these questions by synthesizing novel substituted porphyrin complexes (fig.1) and using a combination of techniques to examine their interaction with DNA.

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2. EXPERIMENTAL

2.1. *Synthesis of porphyrins and metalloporphyrins*

The precursor complex, *meso*-tetrapyridylporphyrin (**5a**) and its Cu, Ni and Mn derivatives (**5b-5d**) used in the synthesis of **1a-1d** and **2a-2d** (see below) were prepared by standard methods [6,7]. **5a** was purified by silica and alumina gel chromatography and refluxed with the appropriate metal acetate in acetic acid to obtain **5b-5d**. Incorporation of metal was checked by UV-vis spectroscopy [6], acetic acid was distilled off, residues were dissolved in $CHCl_3$, washed with aqueous ammonia and water, and recrystallized from $CHCl_3$/heptane.

Porphyrin **1a** and its metal complexes **1b-1d** were made by refluxing **5a**, **5b**, **5c**, or **5d** in ethylene chlorohydrin for 0.5-2 h [8]. Solvent was removed by distillation and the residue washed with $CHCl_3$ and diethyl ether. (The Mn complex, **5d**, was then dissolved in water and treated with Dowex 1-X8 (Cl^- form) ion exchange resin.) The resulting porphyrins **1a-1d** were recrystallized from water/1-propanol (1:9 v/v). Compounds **2a-2d** were prepared by methylating the parent porphyrins **5a-5d** with methyl *p*-toluenesulphonate [9]. The porphyrins were passed through a Dowex 1-X8 (Cl^- form) ion exchange column to substitute a Cl^- counterion, and then purified as for **1a-1d**.

Porphyrins **3** and **4** were derived from the parent 5,10,15-triphenyl-20-pyridylporphyrin (**6**) which was obtained by fractionating the porphyrin products carrying different ratios of phenyl:pyridyl substituents arising when a mixture of benzaldehyde and isonicotinaldehyde is condensed by refluxing in proprionic acid. Compound **6** was isolated by column chromatography on silica gel using gradient elution with acetone and toluene, and its structure confirmed by ^{1}NMR spectroscopy. Reaction of **6** with ethylene chlorohydrin or

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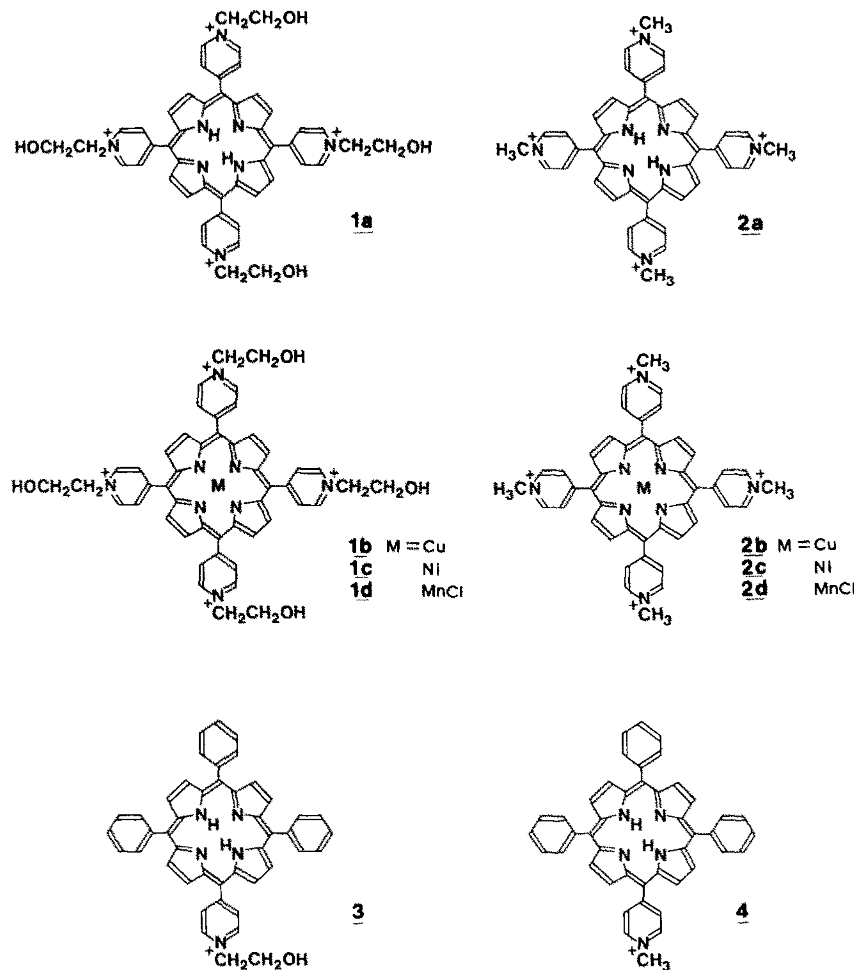

Fig.1. Structures of porphyrins used in this study.

methyl *p*-toluenesulphonate as described above yielded compounds **3** and **4**, respectively.

2.2. *DNA binding and intercalation by porphyrins monitored by DNase I footprinting, DNA melting and DNA helix unwinding*

For DNase I footprinting studies, a 143 basepair (bp) *Hin*dIII-*Sau*96I DNA restriction fragment from pBR322 (nucleotide positions 29–172) was purified on low gelling agarose and ^{32}P-labeled at its 3′ *Hin*dIII end using [a-^{32}P]dATP and reverse transcriptase [10]. The reaction mixture (10 μl) contained 10 mM Tris · HCl pH 7.5, 10 mM NaCl, end-labelled DNA, and porphyrin complex (2–4 μM). The solution was preincubated at 30°C for 15 min and then DNase I (Sigma) (2 μl of a 7200 U/μl stock in 150 mM NaCl, 1 mM $MgCl_2$ freshly diluted 1.5×10^5 fold in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$) was added and incubation continued at 30°C. DNA samples (2.5 μl) were removed at 1, 5 and 30 min and added to 3 μl of stop buffer (80% formamide, 10 mm EDTA, 0.1% bromophenolblue). Samples were heated to 90°C for 1 min prior to loading and electrophoresis on a denaturing 10% polyacrylamide sequencing gel. Autoradiography and Maxam-Gilbert sequencing reactions were done as described previously [11]. DNA melting curves were determined in 250 μM sodium phosphate buffer, pH 6.7, containing NaCl (final ionic strength 20 mM), 40 μM sonicated calf thymus DNA and 2 μM porphyrin complex (i.e. r_o = 0.05). Measurements were made on a Beckman DU64 instrument equipped with temperature controller and thermocouple monitor using a temperature rise of <0.5 deg/min. 1-Propanol (final concentration of 0.4% v/v) was added for **3** and **4** to ensure their solubility in water. Unwinding of closed circular pBR322 DNA (2.5 μg) by porphyrins was detected by incubation with calf thymus DNA topoisomerase I (20 units, final reaction volume of 100 μl) by a method described previously [12]. DNA unwinding experiments on **3** and **4** were carried out in buffers containing 20% DMSO, in which both compounds are soluble and DNA topoisomerase I remains active.

3. RESULTS

3.1. *DNase I footprinting of porphyrins with +4 charges but different pyridiniumyl sidechains*

The effects of sidechain substitution on the binding of +4 charged *meso*-substituted porphyrins to DNA were examined by DNase I footprinting (fig.2). Binding of *meso*-tetrakis[4-*N*-(2-hydroxyethyl)pyridiniumyl]-porphyrin and its Cu and Ni complexes (**1a–c**) gave similar footprints (fig.2a) with a characteristic pattern of protected and enhanced sites of DNase I digestion (fig.2A,B). Despite the presence of the more bulky 2-hydroxyethyl group with its potential for hydrogen bonding interactions, the footprinting patterns for **1a–c** were very similar if not identical to that of porphyrin **2a**

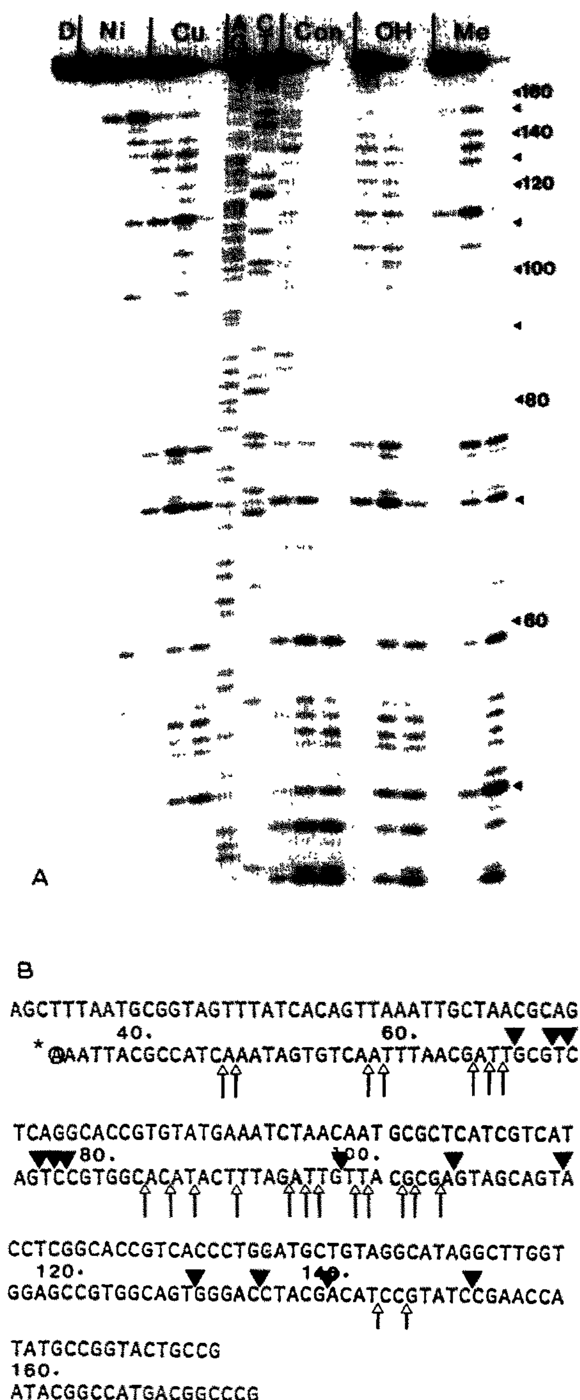

Fig.2. Site-specific binding of +4 positively charged porphyrins to DNA revealed by DNase I footprinting. (A) A *Hin*dIII-*Sau*96I DNA restriction fragment from pBR322 3′ labeled at the *Hin*dIII end was digested with DNase I for 1, 5 and 30 min in the absence (Con) or presence of porphyrin or metalloporphyrins each at 2 μM: OH, Cu and Ni denote compounds **1a–c**; Me denotes **2a**; D is untreated DNA. G+A, C+T denote Maxam-Gilbert sequencing products for the fragment. Numbers refer to nucleotide positions in the pBR322 sequence [Sutcliffe, 1979]. (B) Summary of footprinting data for the labelled DNA strand. Asterisk denotes labelled 3′ DNA end; open and filled arrows denote sites of diminished and enhanced DNase I attack in the presence of porphyrins, respectively.

which bears methylpyridiniumyl sidechains. All these porphyrins gave protection against nuclease attack at both CG and AT sequences (fig.2b). Porphyrins **3** and **4** at 2 or 4 μM did not produce a DNase I footprint (results not shown).

3.2. *DNA helix stabilization by porphyrins: side-chain alteration does not affect the reduced affinity of Mn porphyrin complexes for DNA*

Fig.3a shows DNA melting curves determined in the presence of **1a** and its Cu (**1b**) and Mn (**1d**) complexes. Mn complexes carrying axial ligands bind DNA by a non-intercalative mechanism and accordingly **1d** displayed only weak helix stabilization. Compounds **1a** and **1b** gave greater helix stabilization suggesting a strong (intercalative) interaction with DNA in line with the DNase I footprinting results. Compound **2a** and its Mn complex **2d** showed identical curves (not shown) to those of **1a** and **1d**, respectively. **1d** and **2d** exhibited no red shift and minor (8–20%) hypochromicity of the porphyrin Soret band (ca. 460 nm) on titration with DNA. This behaviour contrasts with titration curves exhibiting an isosbestic point, 5–11 nm red shift and

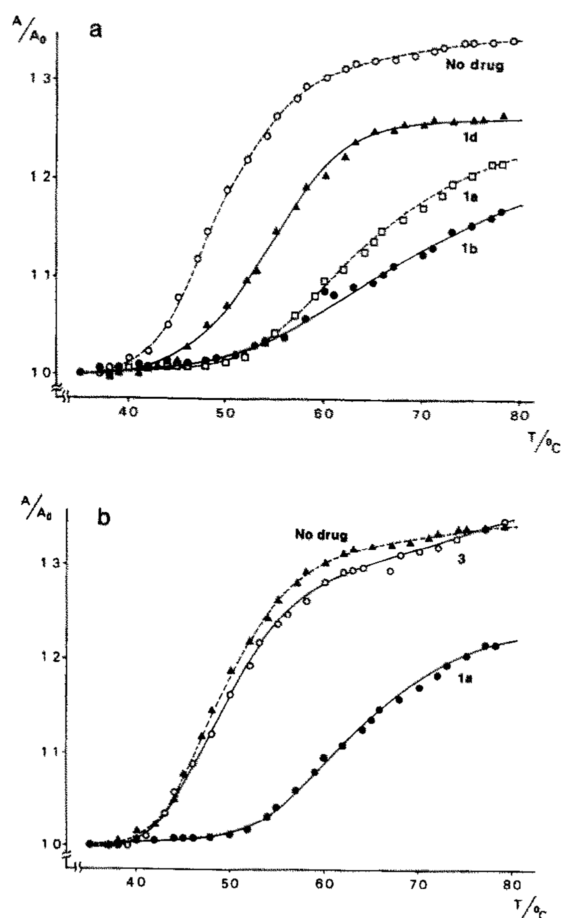

Fig.3. DNA melting curves determined in the presence of porphyrins. (a) Data for +4 positively charged compounds; (b) comparison of helix stabilization by singly and +4 positively charged compounds.

40–55% hypochromicity of the Soret band (ca. 420 nm) observed for **1a**, **1c** and **2a** (not shown), again consistent with intercalative binding by these complexes.

3.3. *DNA helix stabilization and unwinding were not observed for singly charged porphyrin derivatives*

In DNA melting experiments, the singly charged porphyrin **3** had little effect in stabilizing the DNA helix in contrast to porphyrins with +4 charges (fig.3b). The result suggested that if the compound bound DNA it did not intercalate. DNA intercalation causes helix unwinding and therefore we examined the unwinding of closed circular pBR322 DNA by porphyrins following relaxation with the enzyme DNA topoisomerase I (fig.4). Compounds **1a–c** and **2a** each caused DNA unwinding consistent with an intercalative mode of binding for these +4 charged compounds (fig.4A). (All

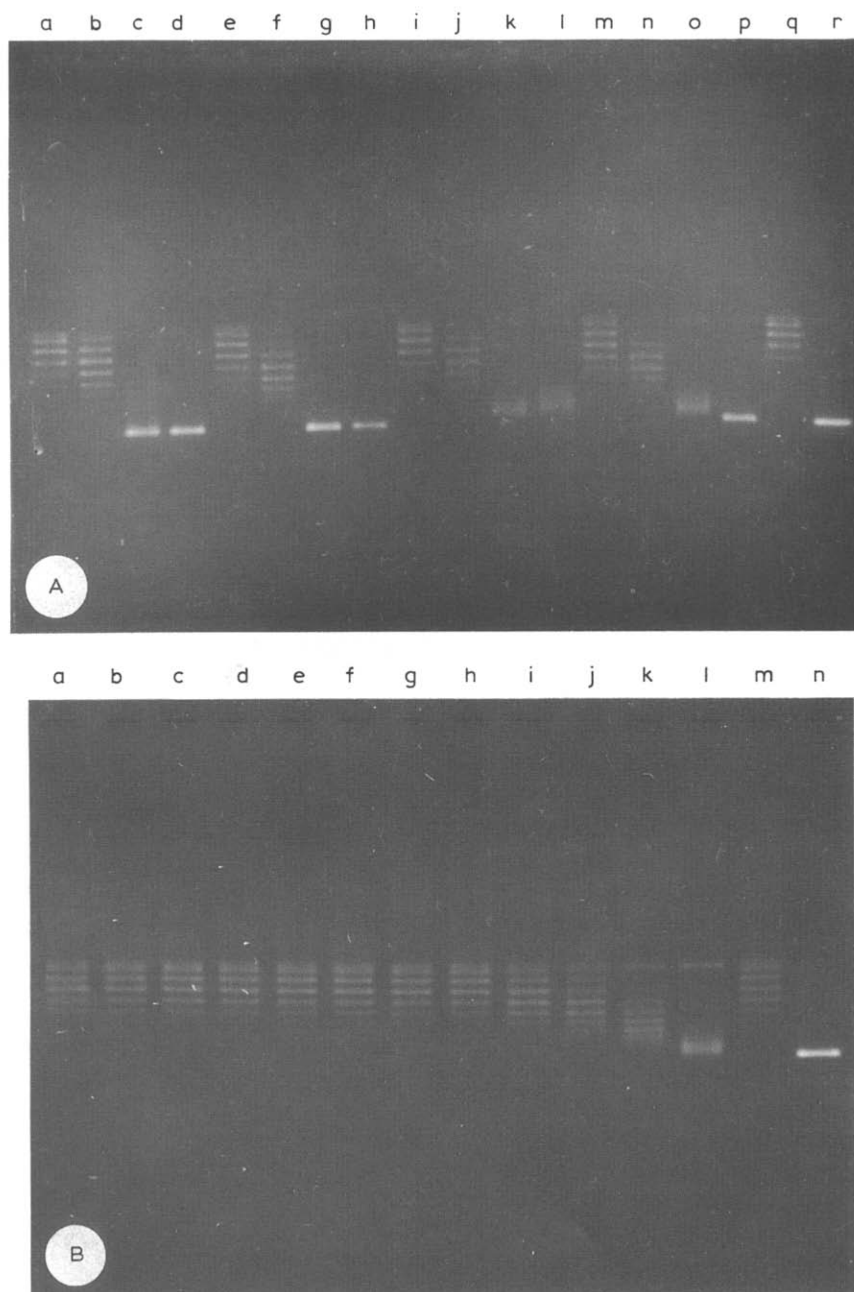

Fig.4. DNA unwinding by porphyrins. (A) +4 charged porphyrins induce unwinding of pBR322 DNA. Supercoiled pBR322 DNA (r) was relaxed by incubation with DNA topoisomerase I and the reaction either terminated (q) or further incubated with porphyrins. Reactions were stopped, the DNA purified by phenol extraction and analyzed by agarose gel electrophoresis in Tris-acetate-Mg^{2+} buffer. Porphyrins **1c**, **1b** and **2a** were included at 0.5, 1, 2.5 and 5 μM in lanes a–d, e–h, and m–p, respectively. Compound **1a** was used at 0.5, 1 and 2.5 μM (lanes i–k). Lane 1 is a partially supercoiled DNA control. (B) Tetrasubstituted +1 charged porphyrins do not promote DNA helix unwinding. Porphyrins **4**, **3** and **1a** were included at 0.5, 1, 2 and 4 μM in lanes a–d, e–h and i–l, respectively. Lanes **m,n**; relaxed and supercoiled DNA controls as above.

topoisomers in fig.4 were shown to be negatively supercoiled (unwound) rather than positively supercoiled by virtue of their increased electrophoretic mobility at 4°C relative to nicked circles [12].) In contrast, neither of the tetrasubstituted monocharged complexes **3** or **4** caused detectable unwinding of DNA (fig. 4B). The absence of unwinding was not due to inhibition of topoisomerase I activity by the compounds. Control experiments (not shown) demonstrated that under the unwinding conditions neither **3** nor **4** at 4 μM inhibited relaxation of the supercoiled pBR322 DNA. Secondly, co-addition of compounds **3** or **4** with porphyrin **1a** each at 4 μM under the conditions of fig.4b gave exactly the same level of helix unwinding as that observed for **1a** alone. Thus, topoisomerase I was fully active in the presence of the singly charged porphyrins.

4. DISCUSSION

Intercalation of porphyrins into DNA constitutes a novel type of DNA-ligand interaction. Previous studies of this process have largely centred on *meso*-tetrakis[4-*N*-methylpyridiniumyl]porphyrin (**2a**) and its metal complexes, which are the best characterized porphyrins. These compounds carry four positively charged aromatic sidechains which each adopt a conformation that is not coplanar with the porphyrin ring [14]. The contribution of these sidechains and the structural features responsible for DNA intercalation are not understood.

We have found that longer sidechain substitutions preserving the number of positive charges do not significantly affect either the affinity or the site specificity of porphyrin binding to DNA. Essentially the same DNase I footprinting patterns and unwinding results were seen for complexes carrying four methyl or four hydroxyethyl sidechains (figs. 2–4). Both types of complex give a DNase I footprint comprising an alternating pattern of protected and enhanced sites of nuclease attack (fig.2). Enhanced nuclease cleavage could be due to exclusion of the nuclease from some regions of the DNA by the site-specific binding of the porphyrin [4,5]. Interpretation of protection in molecular terms is more difficult but presumably reflects both local site-specific intercalation or binding and any longer range effects arising from perturbation of the helix. These observations show that the nature of the substituent on the pyridyl ring does not markedly affect the porphyrin DNA interaction.

In contrast, meso-substituted porphyrins, in which only one of the aromatic rings carries a positive charge, did not intercalate into DNA (irrespective of sidechain identity) as judged by DNase I footprinting, DNA helix stabilization or DNA unwinding measurements (figs 2–4). Previously, Sari et al. synthesized the compounds (4-x)phenyl-x(4-methylpyridiniumyl)porphyrin (where x = 2, 3 or 4) [13]. They inferred from fluorescence energy transfer experiments that such derivatives bound and intercalated into DNA and that the porphyrin ring was important for this process. However, unlike the results presented here their experiments did not extend to $x = 1$.

The pathway for the formation of the porphyrin-DNA intercalation complex is not yet understood but presumably involves transient opening of the DNA helix, insertion of the porphyrin ring system between the DNA basepairs and stabilization of the intercalation complex by ionic interactions between the positively charged compound and the DNA backbone. For porphyrins such as 3 and 4 carrying only a single positively charged substituent, the electrostatic interactions with DNA may be insufficient to anchor and stabilize the intercalated ligand, thereby accounting for their low affinity for DNA. Hardly any difference in DNA binding was observed for +4 charged compounds with two different sidechains. The results indicate an orientation of the porphyrin ring within the DNA basepairs such that sidechain alteration can be readily accommodated. Interestingly, molecular graphics studies using energy minimization techniques of **2a** intercalated into CG basepairs do suggest a binding mode in which the pyridiniumyl groups are placed symmetrically into the minor and major grooves [14]. Confirmation of these models awaits the three dimensional structure determination of a porphyrin-DNA intercalation complex.

These studies highlight the role of electrostatic interactions on porphyrin binding to DNA. Such considerations will be important in the design of site-specific DNA binding agents based on porphyrin ring systems.

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