Rotation of Periphery Methylpyridine of *meso*-Tetrakis(*n*-*N*-methylpyridiniumyl)porphyrin (*n* = 2, 3, 4) and Its Selective Binding to Native and Synthetic DNAs

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ABSTRACT By utilizing circular and linear dichroism, the binding mode of *meso*-tetrakis(*n*-*N*-methylpyridiniumyl)porphyrin (*n* = 2, 3, 4) to various DNAs was studied in this work. 2-*N*-(methylpyridiniumyl)porphyrin(*o*-TMPyP), in which rotation of the periphery pyridinium ring is prevented, exhibits similar spectral properties when bound to DNA, poly[d(G-C)$_2$] and poly[d(A-T)$_2$], suggesting a similar binding mode. Close analysis of the spectral properties led us to conclude that *o*-TMPyP sits in the major groove. However, both 3-*N*- and 4-*N*-(methylpyridiniumyl)porphyrin (*m*- and *p*-TMPyP), of which the periphery pyridinium ring is free to rotate, intercalate between the basepairs of DNA and poly[d(G-C)$_2$]. In the presence of poly[d(A-T)$_2$], *m*-TMPyP exhibits a typical bisignate excitonic CD spectrum in the Soret band, while *p*-TMPyP shows two positive CD bands. The excitonic CD spectrum of the *m*-TMPyP-poly[d(A-T)$_2$] complex and the positive CD band of the *o*-TMPyP-poly[d(A-T)$_2$] complex were not affected by the presence of the minor groove binding drug, 4′,6-diamidino-2-phenylindole (DAPI), indicating that this porphyrin is bound in the major groove. In contrast, two positive CD bands of the *p*-TMPyP-poly[d(A-T)$_2$] complex altered in the presence of DAPI. From the changes in CD spectrum and other spectral properties, a few possible binding modes for *p*-TMPyP to poly[d(A-T)$_2$] are suggested.

INTRODUCTION

The interaction of cationic porphyrins with nucleic acids has been a subject of intensive investigation (for review see Pasternack and Gibbs, 1996; Pratviel et al., 1989) since the pioneering work of Fiel and co-workers (Fiel et al., 1979), owing to its potential applications in cancer chemotherapy and antiviral activity.

The three-binding mode for the porphyrin-DNA complex has been generally accepted (Fiel, 1989; Marzilli, 1990), namely intercalation, outside self-stacking, and outside random binding. These structures of the porphyrin-DNA complexes have been extensively characterized using a variety of physical techniques. NMR, equilibrium dialysis, flow dichroism, and viscometry measurements on oligo and polynucleotides have supported that porphyrins intercalate into GC-rich regions and that they bind in an outside manner at AT sites (for review see Fiel, 1989; Strickland et al., 1990). Subsequent NMR study has shown that the intercalation occurs only at the 5′CG3′ site, not at 5′GC3′ or other sites (Marzilli et al., 1986; Guliaev and Leontis, 1999). Compared to the intercalation mode, outside self-stacking and outside random binding are less well-characterized. Porphyrins that exhibit an outside self-stacking mode include *meso*-tetrakis(*p*-tri-*N*-methyl-pyridiniumyl)porphyrin (Marzilli et al., 1986; Banville et al., 1986; LeDoan et al., 1987), *meso*-tetrakis[4-[(3-(trimethylammonio)prophyloxy) phenyl]porphyrin (TθOPP) (Mukundan et al., 1994, 1995), *trans*-bis(*N*-methylpyridinium-4-yl)diphenyl porphyrin (Pasternack et al., 1998; Ismail et al., 2000), and some copper(II) porphyrins (Pasternack et al., 2001). These porphyrins stack outside DNA and induce DNA aggregation. The outside random binding modes of porphyrins were also reported (Banville et al., 1986; Carvlin and Fiel, 1983). In this binding mode, porphyrins interact with the phosphate group of DNA through electrostatic interaction. This binding mode is in competition with intercalation. Exceptions in the binding mode have also been suggested for various porphyrins (Kuroda and Tanaka, 1994; Sehlstedt et al., 1994; Schneider and Wang, 1994; Lipscomb et al., 1996; Yun et al., 1998; Barnes and Schreiner, 1998). When Cu(II) [*meso*-tetra(*N*-methyl-4-pyridyl)porphyrin] forms a complex with hexamer duplex [d(CGATCG)]$_2$, porphyrin intercalates: the cytosine base of 5′CGA3′ flips out from DNA (Lipscomb et al., 1996). This result from an x-ray structure was criticized later by NMR study (Barnes and Schreiner, 1998). The groove binding mode, mainly based on the extensive circular dichroism (CD) and linear dichroism (LD) study was also suggested (Kuroda and Tanaka, 1994; Sehlstedt et al., 1994; Schneider and Wang, 1994; Lipscomb et al., 1996; Yun et al., 1998). The side of the porphyrin ring fits into the minor groove of DNA or locates in the major groove by electrostatic interaction between the negatively charged phosphate group of DNA and the positively charged pyridinium ring of porphyrin.

The binding mode could be modulated by the nature of the metal ion and the size and location of the substituent groups on the periphery of the porphyrin. Generally, the free bases and square planner complexes such as Ni^{2+} and Cu^{2+} intercalate between DNA basepairs (to the GC site). For the porphyrin-metal complex, having axially bound ligands such as Co^{3+}, Mn^{3+}, and Fe^{3+} or those with bulky sub-

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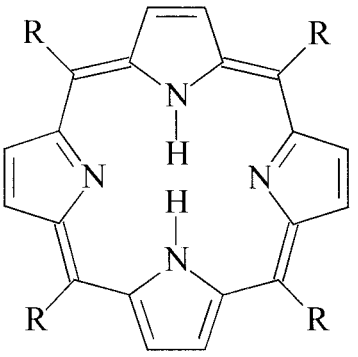

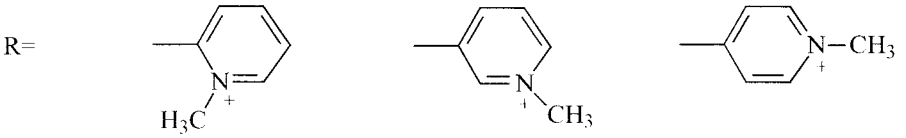

FIGURE 1 Molecular structures of *meso*-tetrakis(*n*-*N*-methylpyridiniumyl)porphyrins ($n = 2, 3, 4$).

stituents on the periphery on the structure, intercalation is blocked and "outside binding" occurs. Recent studies showed that intercalation versus outside binding may also be influenced by the charge on the porphyrin core (Kuroda et al., 1990; Marzilli et al., 1992) and the ionic strength of the medium, which affects self-association of the porphyrin (Pasternack et al., 1993; Dixon and Steullet, 1998). For instance, when the *n*-butyl group is attached to the periphery pyridinium ring, porphyrin intercalates between the DNA basepairs (Sehlstedt et al., 1994), while when replaced by the *tri*-methyl group, porphyrin exhibits an outside self-stacking binding mode (Banville et al., 1986), therefore indicating the importance of the steric effect of the periphery pyridinium ring.

In this work we systematically investigated and classified the binding geometry of *meso*-tetrakis(*n*-*N*-methylpyridiniumyl)porphyrin (where $n = 2, 3, 4$; Fig. 1; referred to as *o*-TMPyP, *m*-TMPyP, and *p*-TMPyP, respectively) to natural calf thymus DNA (referred to as DNA) and poly[d(A-T)$_2$] and poly[d(G-C)$_2$] at a low porphyrin-to-DNA base ratio using conventional spectroscopic methods including CD and LD. The goal of this work is to study the influence of steric hindrance of the periphery pyridinium ring on porphyrin-DNA binding. The rotation of the periphery pyridinium ring of *o*-TMPyP requires high energy, and was expected to prevent porphyrin intercalation (Carvlin et al., 1982; Fiel, 1989; Marzilli, 1990), while this moiety is free to rotate without any hindrance in *m*- and *p*-TMPyP. The porphyrin-to-DNA base ratio was kept very low (lower than one porphyrin molecule per 20 nucleobases) in this work because porphyrin is expected to exhibit a homogeneous binding mode that results in well-defined spectral properties at low mixing ratios (Sehlstedt et al., 1994; Yun et al., 1998). Poly[d(A-T)$_2$] and poly[d(G-C)$_2$] were chosen because these two polynucleotides exhibit the two representative binding modes for *p*-TMPyP, namely intercalation between the GC basepair and outside binding at the AT-rich site. From this study we also hope to relate the known "outside stacking" and "outside random binding" to recently suggested minor or major groove binding (Kuroda and Tanaka, 1994; Sehlstedt et al., 1994; Schneider and Wang, 1994; Lipscomb et al., 1996; Yun et al., 1998).

MATERIALS AND METHODS

Materials

Porphyrins were purchased from Midcentury (Chicago, IL) and used without further purification. The extinction coefficients for the *o*-TMPyP, *m*-TMPyP, and *p*-TMPyP were determined spectrophotometrically to be, respectively, $\epsilon_{413\ nm} = 2.39 \times 10^5\ cm^{-1}\ M^{-1}$, $\epsilon_{417\ nm} = 2.78 \times 10^5\ cm^{-1}\ M^{-1}$, and $\epsilon_{421\ nm} = 2.45 \times 10^5\ cm^{-1}\ M^{-1}$ in 5 mM cacodylate buffer at pH 7.0. Synthetic polynucleotides were purchased from Pharmacia (Seoul, Korea) and DNA from Sigma (Seoul, Korea). Synthetic polynucleotides and DNA were dissolved in 5 mM cacodylate buffer, containing 100 mM NaCl and 1 mM EDTA at pH 7.0 by exhaustive stirring at 4°C, followed by dialyzing against 5 mM cacodylate buffer, pH 7.0 at 4°C. The buffer was changed six times at 5-h intervals and this buffer was used throughout this work. The DNA concentrations were determined spectrophotometrically using molar extinction coefficients: $\epsilon_{258\ nm} = 6700\ cm^{-1}\ M^{-1}$, $\epsilon_{262\ nm} = 6600\ cm^{-1}M^{-1}$, $\epsilon_{254\ nm} = 8400\ cm^{-1}\ M^{-1}$ for DNA, poly[d(A-T)$_2$], and poly[d(G-C)$_2$], respectively. The DNA concentrations given in this work thus indicate the concentration of the nucleobases. The mixing ratio, *R*, is defined by the ratio [porphyrin]/[nucleobase]. The samples with various *R* ratios were prepared by adding aliquots of concentrated porphyrin solution to DNA solution (typically 10–20 μl porphyrin solution to 2

ml DNA solution) and the volume corrections were made. Because the binding mode is affected by ionic strength and porphyrin-DNA mixing ratio and stacking of porphyrin itself in solution (Ismail et al., 2000), an extreme caution for the order of mixing and the concentration of porphyrin stock solution was taken. All measurements were performed at an ambient temperature.

Absorption and circular dichroism

Absorption spectra were recorded either on a Jasco V-550 or on a Hewlett Packard 8452A diode array spectrophotometer using a 1 cm quartz cell. Porphyrins do not possess any chiral center, but acquire an induced CD spectrum upon binding to polynucleotides. The origin of induced CD for the achiral drug-DNA complex, which appears in the drug absorption region, is primarily an effect of coupling between the transitions of drugs and the bases of the nucleic acid host (Lyng et al., 1991, 1992). CD spectra were measured on a Jasco J-715 spectropolarimeter (displaying the CD in millidegrees ellipticity) using a 1-cm cell. The CD spectrum was averaged over an appropriate number of scans when necessary. The CD spectrum of the *m*-TMPyP–poly[d(A-T)$_2$] complex was recorded right after mixing (only one scan) with extreme caution because it changes with time (see below).

Reduced linear dichroism

Linear dichroism (LD) on the flow-aligned porphyrin-DNA complexes was measured in a Couette cell as described by Nordén and his co-workers (Nordén and Tjerneld, 1976; Nordén and Seth, 1985; Nordén et al., 1992) on a Jasco J-500C spectropolarimeter. The Measured LD spectrum is divided by the isotropic absorption spectrum, A_{iso}, to give LD^r, which is related to an angle, α, between the light-absorbing transition dipole and the local helix axis of DNA.

$$\mathrm{LD}^r(\lambda) = \frac{\mathrm{LD}(\lambda)}{A_{iso}(\lambda)} = \frac{3S}{2}(3\cos^2\alpha - 1)$$

where S is the orientation function such that $S = 1$ for a sample perfectly oriented parallel to the flow direction and $S = 0$ for isotropic orientation (Nordén et al., 1992). An effective angle was calculated from the LD^r value observed for the in-plane $\pi\rightarrow\pi^*$ transitions in the Soret band by assuming an effective angle of 86° at 260 nm for the DNA bases with respect to the flow direction (Matsuoka and Nordén, 1983). A larger or comparative LD^r magnitude in the Soret band compared to that in the DNA absorption region is indicative of intercalation, whereas small α values are consistent with groove binding. In the present case, in-plane $\pi\rightarrow\pi^*$ transitions are degenerated in the Soret band. The tilt angle is then obtained by replacing $\cos^2\alpha$ in the above equation by $\frac{1}{2}(\cos^2\beta)$ (Härd and Nordén, 1986).

RESULTS

Absorption spectra

In general, drug binding, including porphyrin, to DNA produces hypochromism, a broadening of envelope and a red-shift in the drug absorption region. These effects are particularly pronounced for intercalators due to π-π stacking between the aromatic ring of drugs and DNA bases. For the groove binders, a large red-shift in the absorption band usually correlates with a drug's conformational change or drug-drug interaction. The absorption spectra of DNA free *o*-, *m*-, and *p*-TMPyP and those in the presence of DNA, poly[d(A-T)$_2$] and poly[d(G-C)$_2$] in the Soret region are depicted in Fig. 2. When *o*-TMPyP is bound to DNA and poly[d(A-T)$_2$], the absorption maximum shifted from 414 nm to 417–418 nm and 8–9% hypochromism is apparent (Fig. 2 *A*). The changes in the absorption spectrum are similar in the presence of DNA and poly[d(A-T)$_2$]. When bound to poly[d(G-C)$_2$] hypochromism reaches 34%, although the red-shift is similar (3~4 nm). Restriction of the rotation of the periphery pyridinium ring may prevent the intercalation of *o*-TMPyP to polynucleotides and exhibits outside binding or major groove binding (see below). The binding of *o*-TMPyP may be similar in DNA and poly[d(A-T)$_2$], but is different from that of poly[d(G-C)$_2$]. The red-shift and hypochromism are more pronounced in the *m*-TMPyP (Fig. 2 *B*) case: red-shift is 11 nm, 16 nm, and 17 nm (from 417 nm of polynucleotide-free *m*-TMPyP) and hypochromism is 31%, 51%, and 55%, respectively, for the poly[d(A-T)$_2$], poly[d(G-C)$_2$], and DNA. When *p*-TMPyP (Fig. 2 *C*) is bound to poly[d(A-T)$_2$], red-shift is 8 nm (from its polynucleotide-free absorption maximum at 422 nm). Those of poly[d(G-C)$_2$] and DNA are 17 nm and 22 nm. Hypochromism is 11%, 46%, and 47%, respectively, for the poly[d(A-T)$_2$], poly[d(G-C)$_2$], and DNA. The shape of the absorption spectrum of the *m*- and *p*-TMPyP-poly[d(G-C)$_2$] complex is intermediate, but resembles that complexed with DNA more closely than with poly[d(A-T)$_2$], which is in contrast with *o*-TMPyP. However, the absorption spectra of both the *m*- and *p*-TMPyP-DNA complexes are not a simple combination of those complexed with poly[d(A-T)$_2$] and poly[d(G-C)$_2$].

CD spectra

Porphyrin induces CD bands in the Soret absorption region when bound to polynucleotide. Recently, the CD behavior of *p*-TMPyP complexed with various polynucleotides was systematically recorded (Lee et al., 2001) when *p*-TMPyP formed a complex with poly[d(A-T)$_2$] and two positive CD bands were apparent while, in the presence of poly[d(G-C)$_2$], *p*-TMPyP exhibits a negative band in the Soret region. In the *p*-TMPyP-poly[d(A-T)$_2$] complex case the band at longer wavelength was dominant (Lee et al., 2001).

The CD spectrum of *o*-TMPyP bound to DNA and to poly[d(A-T)$_2$] is essentially the same and consists of a strong positive band at 416 nm and a positive shoulder around 400 nm (Fig. 3 *A*), indicating that the interaction of the electric transition moments of porphyrin and those of DNA bases are similar in both complexes. The intensity of induced CD in the same region as the *o*-TMPyP-poly[d(G-C)$_2$] complex is smaller by a factor of ~3 and the maximum is at 413 nm. In contrast, the CD spectra of *m*- (Fig. 3 *B*) and *p*-TMPyP (Fig. 3 *C*) exhibit overall negative CD band(s) when complexed with DNA and poly[d(G-C)$_2$]. The maximum intensity in the negative CD band appears at 427 nm and 428 nm for the *m*-TMPyP-poly[d(G-C)$_2$] and the *m*-TMPyP–DNA complex, while it appears at 441 nm and 436

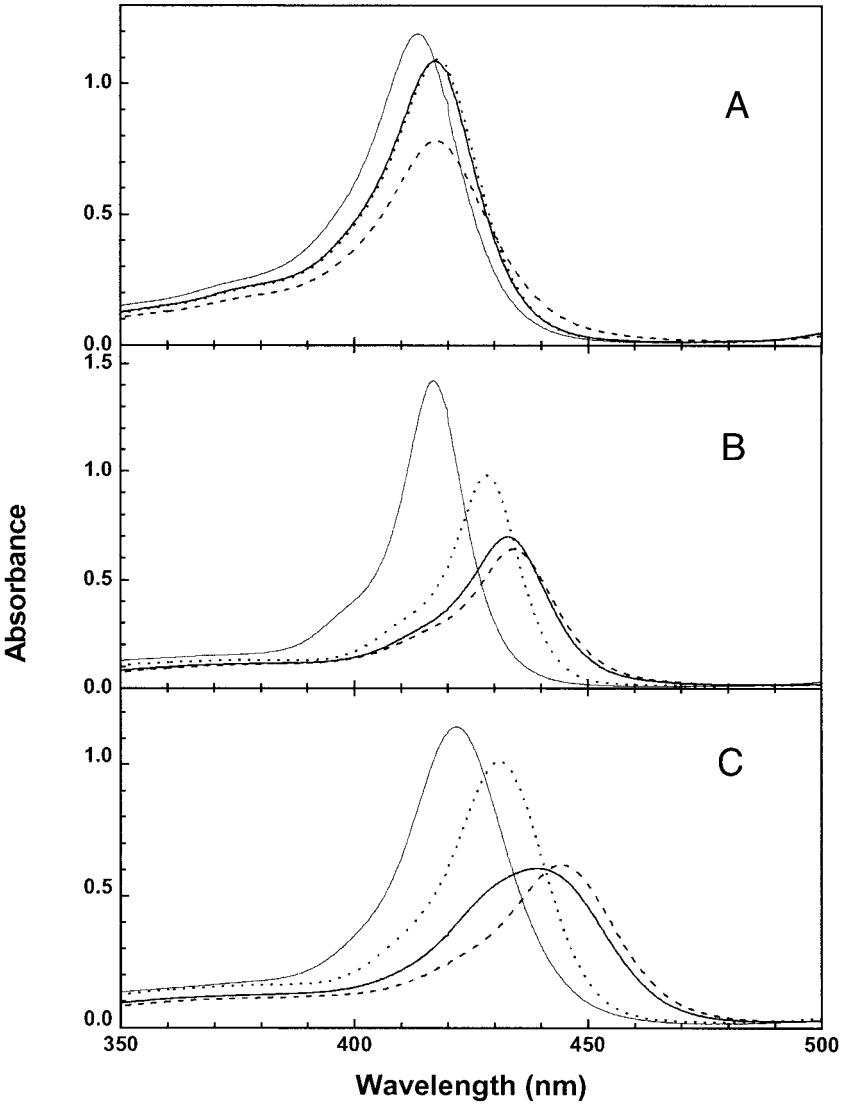

FIGURE 2 (*A*) Absorption spectrum of DNA-free *o*-TMPyP (*thin solid curve*) and that complexed with DNA (*thick solid curve*), poly[d(A-T)$_2$] (*dotted curve*), and poly[d(G-C)$_2$] (*dashed curve*). [DNA] = 101.7 μM, [poly[d(A-T)$_2$]] = 101.7 μM and [poly[d(G-C)$_2$]] = 99.8 μM, and [*o*-TMPyP] = 5.0 μM, [*m*-TMPyP] = 5.1 μM, and [*p*-TMPyP] = 4.7 μM. (*B*) Absorption spectrum of *m*-TMPyP. Curve assignment and concentration is the same as in (*A*). (*C*) Absorption spectrum of *p*-TMPyP. Curve assignment and concentration is the same as in (*A*).

nm for *p*-TMPyP associated with DNA and poly[d(G-C)$_2$]. The *m*-TMPyP-poly[d(A-T)$_2$] complex exhibits a unique CD spectrum (Fig. 3 *B*): the CD spectrum consists of both a strong positive band at long wavelength (maximum at 430 nm), and a strong negative one (minimum at 420 nm), which is typical for excitonic CD observed for the stacked porphyrins. The intensity of excitonic CD decreases and disappears within an hour (see below for change in CD spectrum with time). The *p*-TMPyP-poly[d(A-T)$_2$] complex exhibits two positive bands centered at ~416 nm and ~435 nm (Fig. 3 *C*) as it was reported (32). The shape of the CD spectrum is similar to that observed for the MnTMPyP-poly[d(A-T)$_2$] complex (Kuroda and Tanaka, 1994). Overall, the CD spectrum of the porphyrin with freely rotating methylpyridiniumyl rings is similar (but not identical) to poly[d(G-C)$_2$] and DNA (Fig. 3, *B* and *C*), whereas *o*-TMPyP, in which the rotation of the periphery pyridine ring is blocked, exhibits the identical CD spectra for the DNA and poly[d(A-T)$_2$] complex (Fig. 3 *A*).

Mixing ratio dependence of absorption and CD spectrum

It is well known that the binding mode of porphyrins to various polynucleotides depends on the mixing ratio (*R* ratio is the ratio of the concentration of porphyrin to DNA base). However, at a mixing ratio below 0.1, the spectroscopic properties are independent of the *R* ratio, indicating that the binding mode at this low *R* ratio is homogeneous (Sehlstedt et al., 1994; Lee et al., 2001). In this condition, the absorption and CD spectra are expected to obey the Beer-Lambert law. The CD spectrum of the *m*-TMPyP complexed with poly[d(G-C)$_2$] and poly[d(A-T)$_2$] is depicted as examples in Fig. 4, *A* and *B*).

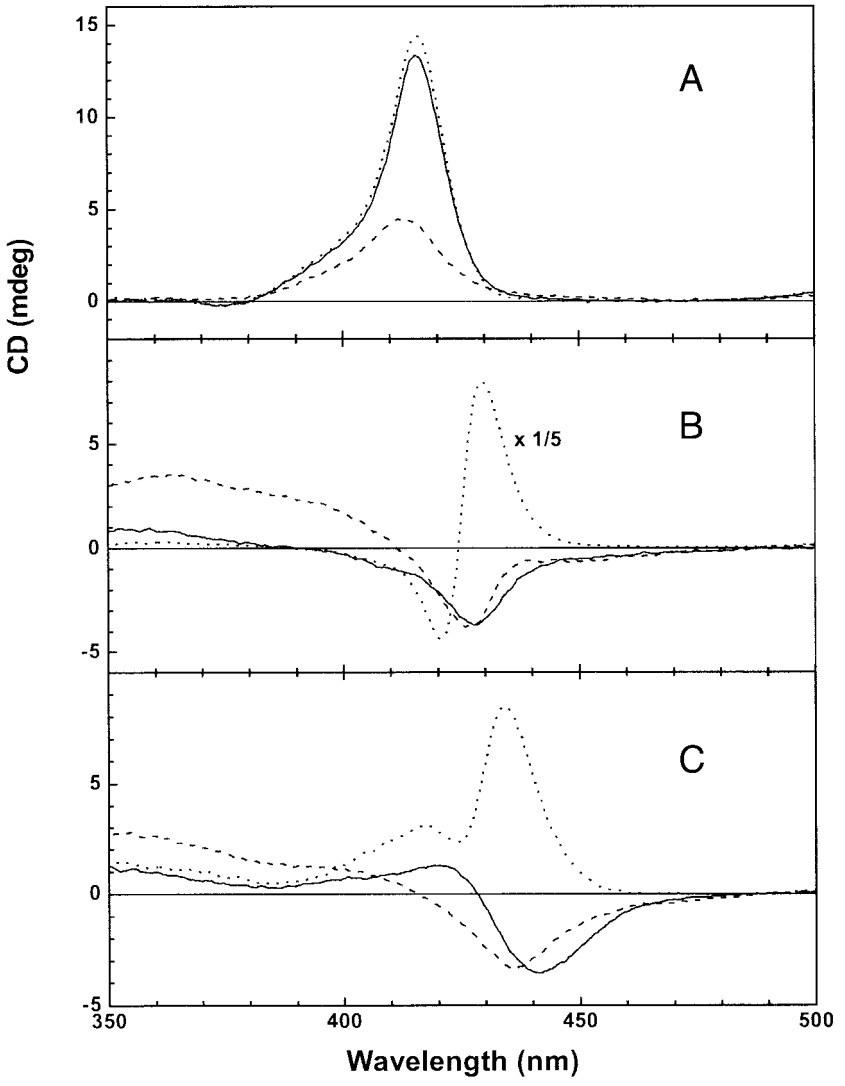

FIGURE 3 CD spectrum of (*A*) *o*-, (*B*) *m*-, and (*C*) *p*-TMPyP complexed with various DNAs. Curve assignment and concentrations are the same as in Fig. 2. In (*B*), CD spectrum of the *m*-TMPyP-poly[d(A-T)$_2$] complex is reduced by the factor of 5 for ease of comparison.

The shapes of CD spectra of these complexes are identical at various R ratios ($R < 0.05$). The absorbance and CD intensity are proportional to the concentration of porphyrin (Fig. 4, *A* and *B*, *insets*). Other complexes exhibit similar independency of R ratio (data not shown), indicating the binding mode at these low R ratios is homogeneous.

Reduced linear dichroism

Reduced linear dichroism (LD^r) is directly related to the angle between the electric transition moment of the polynucleotide-bound drug and the DNA helix axis. This property makes LD^r very useful to determine the binding mode of drug relative to the polynucleotides (Nordén et al., 1992) and has already been applied to some of the porphyrin-DNA complexes (Geacintov et al., 1987; Sehlstedt et al., 1994; Yun et al., 1998; Lee et al., 2001).

At a glance, the magnitude of LD^r in the Soret band is larger than that of the DNA absorption region for the *m*- and *p*-TMPyP complexed with DNA (Fig. 5 *C*) and poly[d(G-C)$_2$] (Fig. 5 *B*). The larger LD^r magnitude in the drug absorption region compared to that in the DNA absorption region is generally accepted as an indication of drug intercalation, as it was reported for acridine and ethidium derivatives (Kim et al., 1996; Tuite and Nordén, 1995). When porphyrin is intercalated between the DNA basepairs, elongation and stiffening from unwinding of the DNA helix is expected, hence an increase in the orientability of DNA in the flow, resulting in an increase in the LD^r magnitude in the DNA absorption region compared to that of drug-free DNA. Both the B_x and B_y in-plane electric transition of porphyrins relative to the DNA helix axis are expected to be perpendicular with respect to the DNA helix axis that results in the wavelength-independent (constant) LD^r magnitude in the Soret band. However, both the *m*- and *p*-TMPyP-DNA

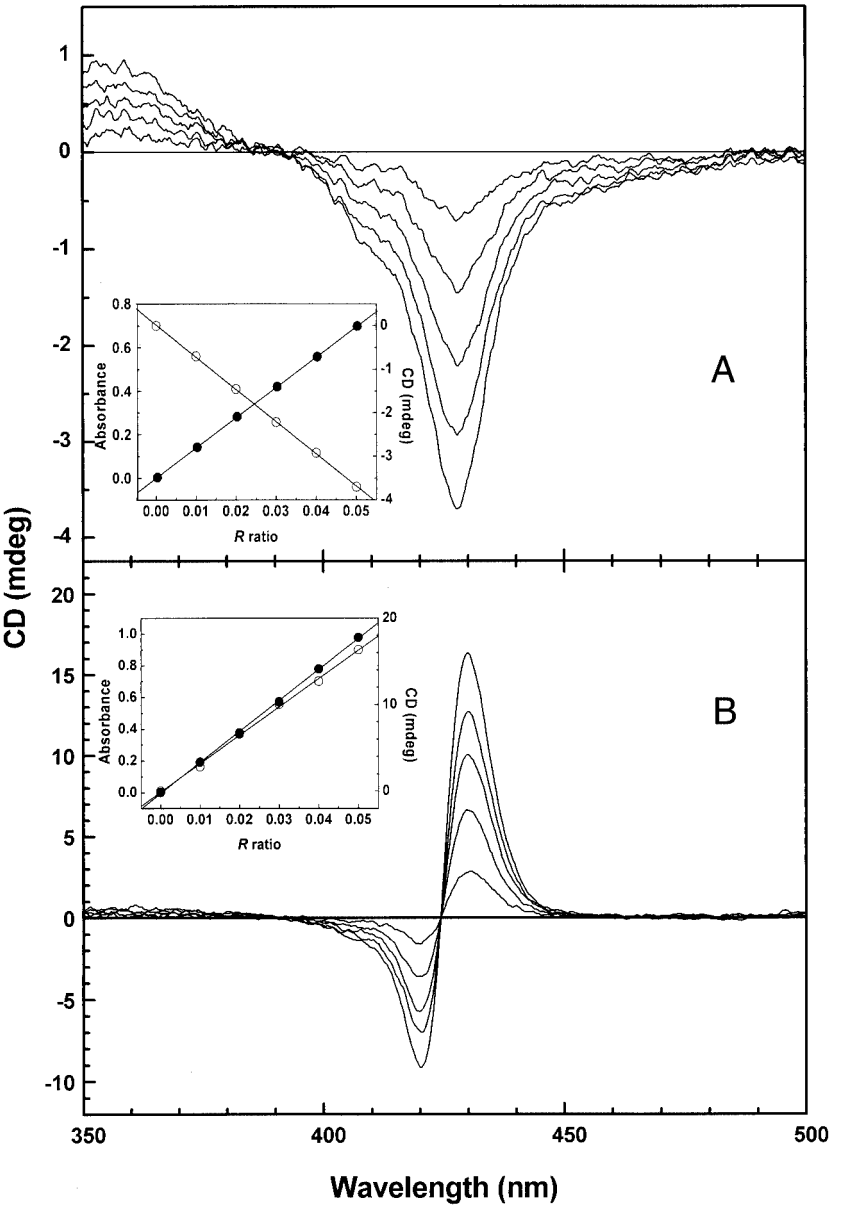

FIGURE 4 CD spectrum of the *m*-TMPyP when complexed with poly[d(G-C)$_2$] (*A*) and with poly[d(A-T)$_2$] (*B*) at various mixing ratios. [Polynucleotide] = 50.0 μM, R = 0.01, 0.02, 0.03, 0.04, and 0.05. *Inset A:* Change in absorbance at 433 nm (*closed circle*) and CD at 428 nm with respect to the R ratio for the *m*-TMPyP-poly[d(G-C)$_2$] complex. *Inset B:* Change in absorbance at 428 nm (*closed circle*) and CD at 429 nm with respect to the R ratio for the *m*-TMPyP-poly[d(A-T)$_2$] complex.

and poly[d(G-C)$_2$] complexes exhibit a wavelength-dependent LD^r magnitude, and the magnitude of LD^r in the DNA absorption regions decreases. This LD^r value indicates that either the flexibilities of DNA and polynucleotides are increased, or they are bent upon intercalation of *m*- and *p*-TMPyP. *o*-TMPyP complexed with all polynucleotides and *m*- and *p*-TMPyP complexed poly[d(A-T)$_2$] exhibit smaller LD^r magnitudes in the Soret band compared to those in the DNA absorption region. Assuming an effective angle of 86° at 260 nm for the DNA bases with respect to the helix axis, the angles α and β were calculated for these complexes and are summarized in Table 1. Although there is no definitive low limit for the angles α and β for intercalation, the angle β, which is lower than 70°, suggests that the possibility of porphyrin intercalation in these complexes can conceivably be ruled out.

CD spectra of the TMPyP-poly[d(A-T)$_2$] complexes when the minor groove is blocked by DAPI

4′,6-Diamidino-2-phenylindole (DAPI) has been well known to bind strongly to the minor groove of poly[d(A-T)$_2$] and covers 4–5 AT basepairs (Nordén et al., 1990; Eriksson et al., 1993), resulting in the narrowing and reduced flexibility at the minor groove. This property was utilized to probe the binding site of porphyrins in this work, particularly for poly[d(A-T)$_2$]. When DAPI blocks the minor groove of

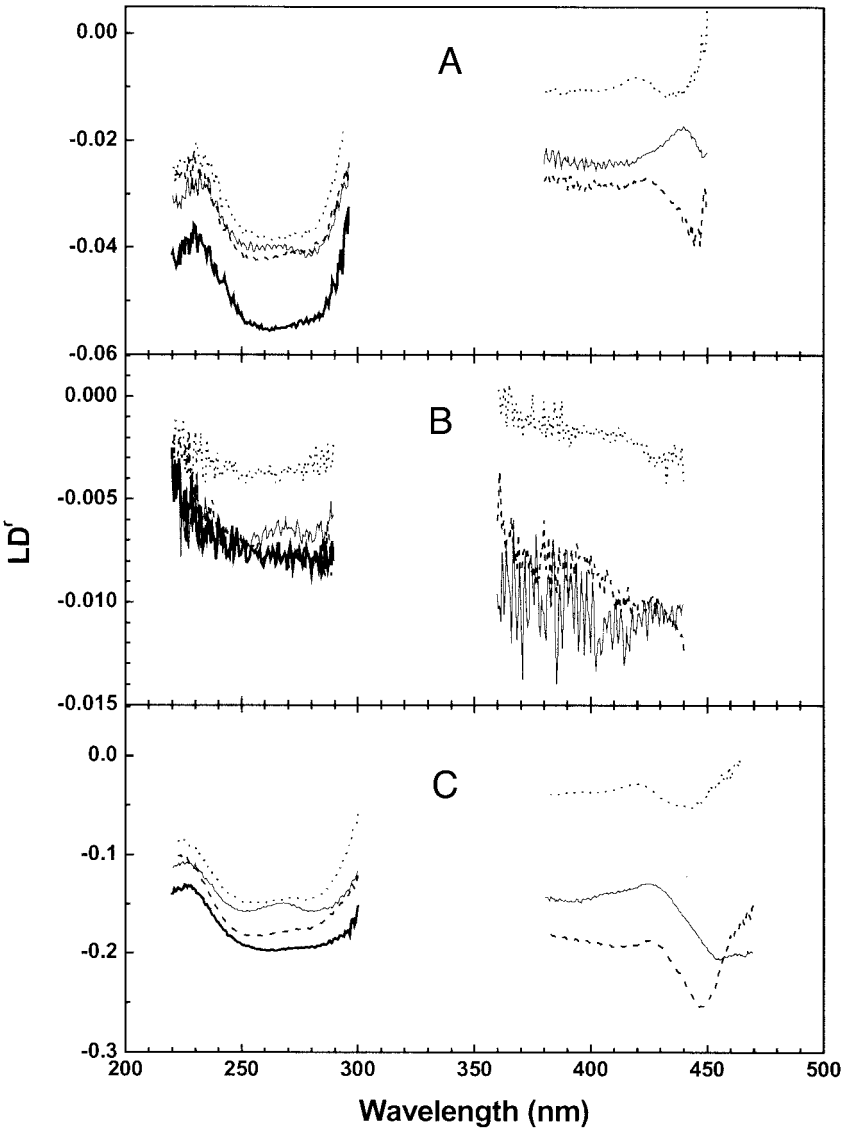

FIGURE 5 Reduced linear dichroism spectrum of (*A*) poly[d(A-T)$_2$], (*B*) poly[d(G-C)$_2$], and (*C*) DNA in the absence (*thick solid curve*) and presence of *o*- (*dotted curve*), *m*- (*dashed curve*), and *p*-TMPyP (*thin solid curve*). [Polynucleotide] = 100 μM, R = 0.05.

TABLE 1 Angle α and β calculated from LD^r

Porphyrin	Polynucleotide	α*	β*
Ortho-TMPyP	DNA	59–62°	43–49°
	poly[d(G-C)$_2$]	69°	60°
	poly[d(A-T)$_2$]	59–61°	44–47°
Meta-TMPyP	DNA	n.d.†	n.d.
	poly[d(G-C)$_2$]	n.d.	n.d.
	poly[d(A-T)$_2$]	70–78°	61–74°
Para-TMPyP	DNA	n.d.	n.d.
	poly[d(G-C)$_2$]	n.d.	n.d.
	poly[d(A-T)$_2$]	65–69°	53–60°

*The angles were calculated from minimum and maximum LD^r values in the Soret band.

†The angles cannot be determined because the magnitude of LD^r in the Soret band is larger than that in the DNA absorption region, which results in an imaginary number in angle calculation. However, these LD^r spectra are often observed for intercalated drugs (see text).

poly[d(A-T)$_2$], spectroscopic properties of the TMPyP are expected to altered in great extent if it is bound in the minor groove, while change would be small if it binds at the surface or major groove of poly[d(A-T)$_2$].

The CD spectrum of the *o*-, *m*-, and *p*-TMPyP-poly[d(A-T)$_2$] complex is compared in the presence and absence of DAPI, respectively, in Fig. 6, *A–C*). Here, the R ratio of DAPI was 0.12, which ensures that all available binding sites in the minor groove are blocked. A strong positive CD band at ~360 nm is apparent when DAPI is complexed with poly[d(A-T)$_2$]. In Fig. 6, the CD spectrum of the poly[d(A-T)$_2$]-DAPI complex was subtracted from the porphyrin-poly[d(A-T)$_2$] complex for ease of comparison. However, it is noteworthy that a strong, positive-induced CD band of DAPI complex remained essentially the same even in the presence of porphyrin, indicating that the binding of porphyrin does not result in the release of DAPI. A positive CD

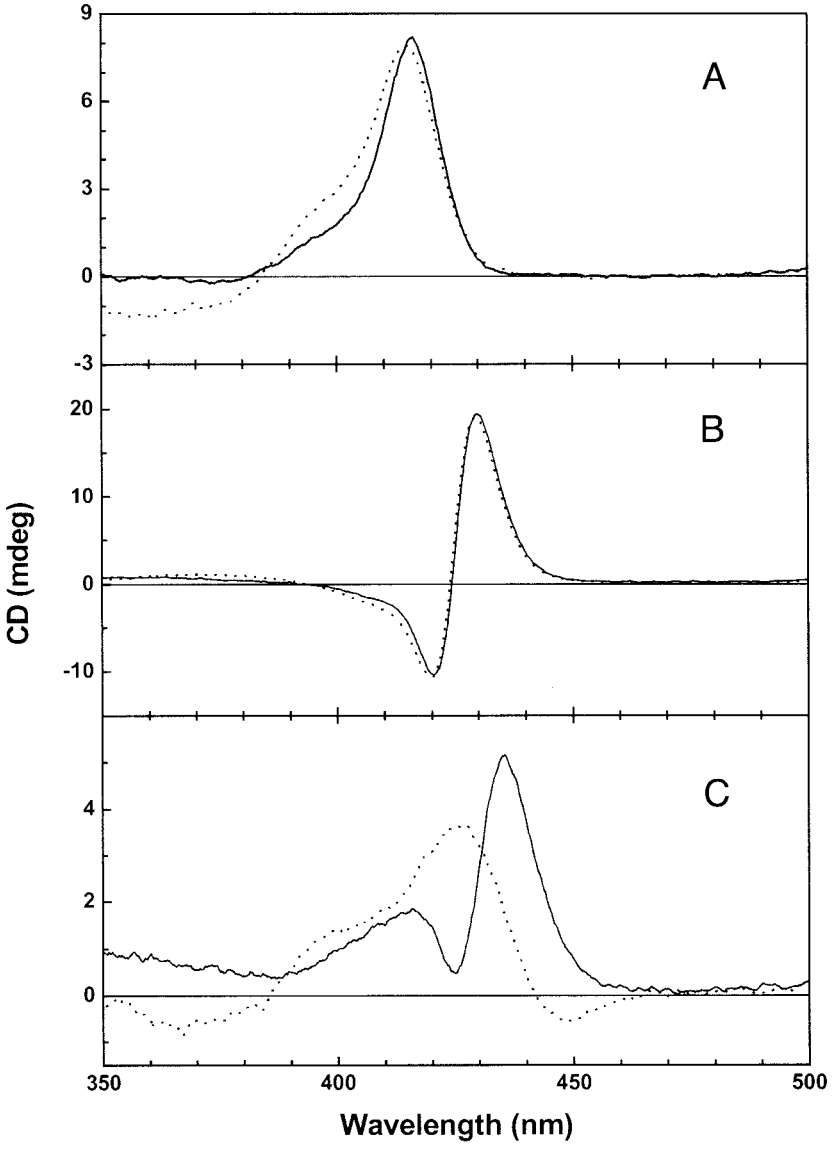

FIGURE 6 CD spectrum of (*A*) *o*-, (*B*) *m*-, and (*C*) *p*-TMPyP complexed with poly[d(A-T)$_2$] in the presence (*dotted curve*) and absence (*solid curve*) of 4′,6-diamidino-2-phenylindole (DAPI). [Polynucleotide] = 50 μM, [DAPI] = 6.0 μM, and [TMPyP] = 2.5 μM. CD spectrum of the poly[d(A-T)$_2$]-DAPI complex was subtracted from that of the TMPyP-poly[d(A-T)$_2$]-DAPI complex for ease of comparison.

band of *o*-TMPyP in the Soret band in the presence and absence of DAPI is similar (Fig. 6 *A*), suggesting that the effect of blocking the minor groove by DAPI to the binding mode of *o*-TMPyP is negligible. However, the CD spectrum of *p*-TMPyP is greatly altered by the presence of DAPI (Fig. 6 *C*): two positive bands at 416 nm and 435 nm blue-shift to ~398 nm and ~425 nm, and a new negative band appears at ~449 nm. For both *o*- and *p*-TMPyP, a new negative band at 360–370 nm is apparent, which may be attributed to partial release of DAPI due to porphyrin binding. The excitonic CD band of *m*-TMPyP remained the same even in the presence of DAPI, indicating that the binding mode of this porphyrin is unaffected. Because the CD spectrum of the *m*-TMPyP-poly[d(A-T)$_2$] complex is time-dependent (see below), the CD spectrum in the presence and absence of DAPI was taken with great care to give the same time interval right after mixing.

Time dependence of the CD spectrum of the *m*-TMPyP-poly[d(A-T)$_2$] complex

The initial CD spectrum of the *m*-TMPyP-poly[d(A-T)$_2$] complex is compared with that after 2 h of mixing in Fig. 7. At this time, the reduction in CD intensity reaches a plateau. The final CD spectrum in the Soret region is symmetrical compared to that of the initial one, although the intensity was reduced to less than a factor of 10. The reduction in CD intensity for the *m*-TMPyP-poly[d(A-T)$_2$] does not correspond to a simple first-order kinetic scheme (Fig. 7, *inset*). Changes in the CD and absorption spectrum in time were reported for TθOPP to DNA and various polynucleotides (Mukundan et al., 1995). However, the time scale for the change of TθOPP (24 h) and *m*-TMPyP-poly[d(A-T)$_2$] (<1 h) is different. Although the unique dynamic aspect of *m*-TMPyP is interesting, further coverage in this di-

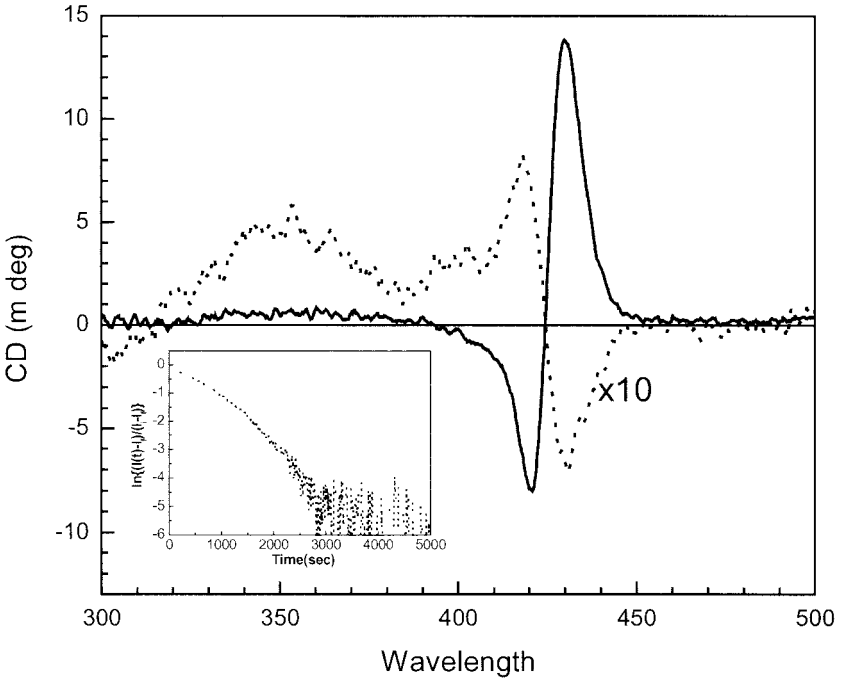

FIGURE 7 CD spectrum in the Soret region of the *m*-TMPyP-poly[d(A-T)$_2$] complex right after mixing and 2 h later. The CD intensity change at 430 nm is plotted as the form of $\ln[(I(t) - I_f)/(I_i - I_f)]$ versus time in seconds to fit in the first-order kinetics (*inset*), where *i* and *f* denote initial and final CD intensity. As shown in the inset, the change is completed in an hour.

rection is out of the scope of this work and will be published elsewhere.

DISCUSSION

Binding sites of *o*-TMPyP

The spectral properties of *o*-TMPyP complexed with polynucleotides are summarized as a small red-shift and hypochromism in the absorption spectrum and positive CD band. The absorption and CD spectra of the *o*-TMPyP-DNA and *o*-TMPyP-poly[d(A-T)$_2$] complexes are almost identical, indicating that this porphyrin prefers to bind to outside of the DNA at a low *R* ratio. The LD^r values for all *o*-TMPyP complexes are negative. The angles of the B_x and B_y in-plane electric transition of porphyrins relative to the DNA helix axis correspond to 43–49° for DNA, 44–47° for poly[d(A-T)$_2$], and 60° for the poly[d(G-C)$_2$] complex relative to the DNA helix axis (Table 1). From these angles it is evident that *o*-TMPyP does not intercalate between the basepairs of DNA because of the steric hindrance of the periphery pyridinium ring, which cannot rotate to form a planar molecule due to the methyl group. An angle of ~45° for any drug that forms a complex with DNA is usually attributed to the minor groove-binding (Nordén and Tjerneld, 1976; Nordén and Seth, 1985; Nordén et al., 1990, 1992; Eriksson et al., 1993; Kim et al., 1996). In the case of porphyrin, it corresponds to the edgewise binding in the minor groove. However, to exhibit this binding mode, planarity of the porphyrin molecule is required because the minor groove is narrow. The *o*-TMPyP cannot be a planner molecule. Furthermore, the blocking of the minor groove by the minor groove-binder DAPI did not significantly alter the CD spectrum. All these factors indicate that the edge of the porphyrin molecule is not inserted in the narrow minor groove disregarding the angle of 45° between the transition moment and the DNA helix axis. Usually, a bisignate CD spectrum in the Soret band is apparent for the outside stacking mode, which is not the case for *o*-TMPyP. Invariance in spectral properties with respect to the *R* ratio (from 0.01) is also against stacking of porphyrin. Rejecting the intercalation, minor groove edgewise-binding, and outside stacking binding modes, the only possible binding modes left over to consider are outside random binding and face-on major groove binding. The possibility of the outside random binding may be ruled out because if porphyrin binds to the phosphate group by electrostatic interaction in a random manner, then the magnitude of LD^r must be zero (LD requires orientation of the sample), which is not the case. The small red-shift and monomeric CD spectrum and the angle of 45° for both B_x and B_y transitions relative to the DNA helix axis do not rule out the last possible binding mode, namely face-on major groove binding, in which one of two lines connecting two periphery pyridinium ring at opposite sides (45° away from both B_x and B_y transition) is parallel to the DNA helix axis and the other perpendicular to that axis. The binding of *o*-TMPyP to poly[d(G-C)$_2$] is less clear. However, the absorption and CD spectrum are similar to those complexed with poly[d(A-T)$_2$] and DNA (although hypochromism is larger and the CD is smaller), suggesting that the binding mode in poly[d(G-C)$_2$] is similar to other polynucleotides. However, the angle β is significantly different, being 60°, indicating that either porphyrin or DNA,

or both, are distorted, or porphyrin is rotated in the major groove. We do not have solid evidence to explain this distortion or rotation at present.

Intercalations of *m*- and *p*-TMPyP to GC-rich regions and native DNA

The optical spectroscopic properties of intercalated *p*-TMPyP to poly[d(G-C)$_2$] and DNA have already been documented (Sehlstedt et al., 1994; Yun et al., 1998). When it is intercalated between the basepairs of DNA or poly[d(G-C)$_2$], large (or larger) LD^r magnitude(s) in the Soret band compared with the DNA absorption region, a negative CD band and large hypochromism and red-shift in the absorption spectrum are produced. From the minimum magnitude of LD^r in the Soret band, the angle β was calculated to be 76° for the *p*-TMPyP complex. The larger LD^r in the drug absorption region compared to that in the DNA absorption region is often observed for intercalators, resulting in an imaginary number for cos α and cos β (Sehlstedt et al., 1994; Tuite and Nordén., 1995; Kim et al., 1996; Yun et al., 1998). This observation was attributed usually to a kink or bend in DNA stem near the drug's binding site. These spectral properties are confirmed for both *p*- and *m*-TMPyP complexed with DNA and poly[d(G-C)$_2$] in this work. At low mixing ratios ($R < 0.05$), spectral properties of both the *m*- and *p*-TMPyP-poly[d(G-C)$_2$] complexes more closely resemble those complexed with DNA than those with poly[d(A-T)$_2$], indicating that these porphyrins prefer to intercalate between the nucleobase of native DNA. However, in contrast with the *o*-TMPyP case, the absorption and CD spectrum of the *m*- and *p*-TMPyP-poly[d(G-C)$_2$] complexes and those complexed with DNA are not identical, indicating that the binding of porphyrin to DNA cannot be explained by the simple combination of AT and GC binding.

Outside binding of *m*- and *p*-TMPyP to AT-rich regions

The shapes of the CD spectra of both *m*- and *p*-TMPyP complexed with poly[d(A-T)$_2$] are quite unique. The *p*-TMPyP-poly[d(A-T)$_2$] complex exhibits two positive CD bands in the Soret region, with intermediate hypochromism and red-shift in the absorption spectrum. Strong negative LD^r, corresponding to the angle β of 61–74°, was also apparent. When the minor groove was blocked by DAPI the CD spectrum changed, which is similar to that observed for the MnTMPyP-poly[d(A-T)$_2$] complex (Kuroda and Tanaka, 1994). The two positive CD bands of MnTMPyP were attributed to the different binding mode, namely major and minor groove binding. This conclusion was based on the observation that intensity of the shorter wavelength peak decreased and that of the longer wavelength increased upon adding beneril or distamycin, which are minor groove binders. In the *p*-TMPyP case, however, instead of a decrease or increase in CD intensity, a blue-shift in the band is more pronounced with a conceivable excitonic CD at the long wavelength edge. Although the change in CD spectra in the presence of DAPI cannot be fully understood, the facts that 1) both CD and absorption spectra are directly proportional to the concentration of added *p*-TMPyP (which supports the homogeneous binding mode); 2) the shape of CD spectrum changes rather than disappears (which is against the edge of porphyrin inserting fully into the minor groove because in this case replacement is expected); 3) the long side chain, such as the *n*-octyl group on the periphery of pyridine did not alter spectral properties (Sehlstedt et al., 1994; Yun et al., 1998) (if the porphyrin is inserted edgewise from the minor groove, the side chain is expected to prevent binding, resulting in the alteration in spectral properties); and finally 4) the angle β (61–74°) being far greater than 45° (which is expected from the porphyrin edgewise insertion to minor groove) suggest that *p*-TMPyP conceivably locates near the minor groove, but is not inserted from there. It seems to be bound at the outside of poly[d(A-T)$_2$] near the minor groove. However, it is equally possible that *p*-TMPyP binds at the major groove and changes its CD spectrum by the conformation change of the polynucleotide due to DAPI binding in the minor groove. From the simple CD result that we observed here, the possibility of the coexistence of major and minor groove binding porphyrins (Kuroda and Tanaka, 1994) also cannot be ruled out.

An excitonic CD is apparent for the *m*-TMPyP-poly[d(A-T)$_2$] complex, indicating that *m*-TMPyP is stacked outside of poly[d(A-T)$_2$]. The presence of DAPI did not affect the CD spectrum. Therefore, it is clear that *m*-TMPyP stacks at the location where the change in minor groove has no effect. Among the known binding modes, stacking at the major groove is most conceivable. The angle β is calculated to be 53–60°, indicating that the plane of the porphyrin molecule is not parallel to DNA basepairs (not perpendicular to the helix axis). Stacked *m*-TMPyP seems to be tilted in the major groove. A change in the position of the methyl group at the pyridine ring from a *para*- to *meta*-position results in a very different binding mode, particularly when complexed with poly[d(A-T)$_2$]. This variation in binding mode cannot be explained by the ability of rotation of the pyridine moiety because the pyridine ring can rotate freely in both the *m*- and *p*-TMPyP case.

CONCLUSIONS

A systematic optical spectroscopic study for *o*-, *m*-, and *p*-TMPyP complexed with poly[d(A-T)$_2$], poly[d(G-C)$_2$], and DNA led us to the following conclusions: 1) preventing the rotation of the periphery methylpyridine ring alters the binding mode, as expected; *o*-TMPyP locates at the major groove; 2) both *m*- and *p*-TMPyP intercalate to poly[d(G-C)$_2$] and DNA at a low *R* ratio; and 3) *m*-TMPyP is stacked

in the major groove of poly[d(A-T)$_2$], while various binding modes can be suggested for *p*-TMPyP.

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