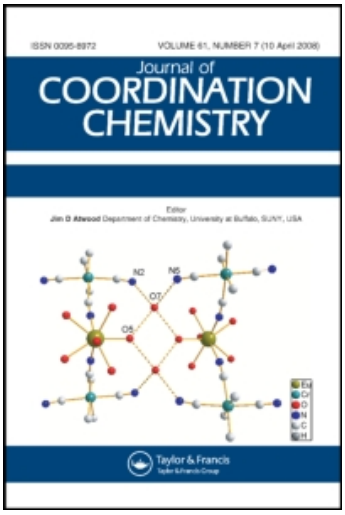

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SALT DEPENDENCE OF BINDING OF Δ– AND Λ–Ru(2, 2′–bipyridine)$_2$ppz (+2) AND A CHIRAGEN ANALOG WITH CALF THYMUS DNA: UNPREDICTIBILITY OF ENANTIOSELECTIVE BINDING TO DNA*

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Fluorescence techniques have been used to measure binding constants with calf thymus DNA for both enantiomers of Ru(bpy)$_2$ppz(+2) as well as a (+)-chiragen[6] complex of ruthenium(II) of Δ configuration, which has ppz as a third ligand. The configuration of the more strongly binding enantiomer of Ru(bpy)$_2$ppz(+2) is unambiguously assigned as Λ (and not Δ as previously assumed), by comparison of the circular dichroism spectrum of the (+)–chiragen[6] complex of ruthenium(II) with ppz to the CD spectra of the bipyridine complexes. This assignment is also consistent with exciton theory of the circular dichroism spectra of such complexes. Binding constants for each enantiomer as well as the (+)-chiragen[6] complex are reported, and serve to quantitate the chiral discrimination of binding to DNA. Analysis of the sodium ion concentration dependence of the binding constants using polyelectrolyte theory indicates that the binding is largely electrostatic in nature. The non-electrostatic contribution (K_t^0) at 50 mM Na^+ is about 3.5% for both isomers and 5.3% for the (+)-chiragen[6] complex. Similar values have been reported for the enantiomers of Ru(phen)$_3$(+2). The approximately four-fold difference in binding between the enantiomers and favoring the Λ (not the Δ) enantiomer reported here must therefore be attributed to differences in structural interaction which are non-electrostatic in nature. Clearly, the configuration of the two ancilliary ligands is not sufficient to predict which enantiomer will bind more strongly, as has been suggested. More subtle interactions with the double strandedB-form DNA structure, involving not only the ancilliary ligands, but also the partially intercalated diimine ligand, are clearly involved.

Keywords: ruthenium trischelate; enantioselective; calf thymus; DNA; salt dependence; chiragen

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Enantioselective binding to double-stranded polynucleotides by tris-chelates of ruthenium(II) and rhodium(III) with diimine ligands has been extensively studied[1–5] for a number of years. Since all such complex ions exist as a pair of enantiomers, binding to chiral double-stranded polynucleotides constitutes a diastereomeric interaction which favors one isomer. The degree to which one isomer is favored has been documented[1–7] and, in general, the Δ isomer appears to be favored in binding to right-handed helices, though to varying degrees. Several exceptions to this have been reported.[5]

For the most studied complex ion, $Ru(phen)_3(+2)$ (phen = 1,10-phenan throline), the preferential binding to calf thymus DNA of the Δ isomer as compared to the Λ isomer has been well documented[3, 4, 7] over a range of Na^+ concentration. The binding constants are small, on the order of 10^4 (n = 3–4) in 50 mM sodium chloride, and binding of the Δ isomer is favored only by a factor of 2 or less. A preference of approximately an order of magnitude for binding of the Δ isomer to an oligonucleotide was observed[6] using NMR techniques, but the complete interpretation indicated a non-intercalative mode of binding within the minor groove.

For the complex ion $Ru(phen)_2DPPZ(+2)$ (DPPZ = dipyrido[3,2-a:2′,3′-c] phenazine) no significant enantioselectivity was observed[3] for binding to calf thymus DNA. The binding constants for both enantiomers in this case were significantly higher (6×10^7). Linear dichroism results were interpreted in terms of inter-calative binding *via* the DPPZ ligand for both enantiomers.

Binding constants of individual enantiomers of $Ru(bpy)_2phi(+2)$ and $Ru(phen)_2phi(+2)$ (phi = 9, 10-phenanthrenequinone diimine) to a series of poly-nucleotides and calf thymus DNA were reported.[5] The values varied from 10^4 to greater than 10^6, with n = 3 to 6. For B-form polynucleotides, the bis-bpy complex showed a significant binding preference (ratio > 1.5) for the Λ isomer in two cases and the bis-phen complex showed a significant preference for the Δ isomer with calf thymus DNA and for the Λ isomer in one case. In six other cases, the preference was less than a factor of 1.5 for either enantiomer.

For a number of complexes involving bpy, phen, and substituted phen, CD signals of dialysates have been interpreted[8] as indicating that the Δ enantiomer binds more strongly to calf thymus DNA.

Enantiomeric preference in binding to polynucleotides must have its basis in steric interactions between the metal complex ion and the chiral double helical DNA. The most favored current interpretation[1–3, 7] of binding for these complexes involves the partial intercalation of one of the diimine ligands within the major groove of the right handed double helix, with the two ancilliary ligands providing a basis for enantiomeric discrimination *via* various

van der Waal's interactions with atoms lining the groove. An early picture of this emerged in which the Δ isomer of $Ru(phen)_3(+2)$ was seen as fitting more readily into the right handed structure. Theoretical basis for this picture has been minimal at best. The generality of this picture must be questioned however as a result of the data presented here which show a four-fold preference for the Λ configuration of $Ru(bpy)_2(ppz)(+2)$ in binding to DNA.

We have reported[1,10] spectroscopic details concerning the binding of individual enantiomers of $Ru(bpy)_2(ppz)(+2)$ (ppz = 4,7-phenanthrolino[6, 5-b]-pyrazine) to calf thymus DNA, leading to the conclusion that both isomers bind *via* partial intercalation of the ppz ligand. In this study, both enantiomers showed hypochromicity of the visible MLCT transition, increased emission intensity, longer emissive lifetimes and polarized emission upon binding to DNA, all supporting an intercalative interaction of the ppz ligand. In contrast, we had previously shown[10] that the complex $Ru(bpy)_2(dpp)(+2)$ (dpp = 2,3-bis-pyridylpyrazine), where the dpp ligand is a non-planar analog of ppz, shows no evidence of binding to DNA. For the study of the ppz complex, the enantiomers were separated on a chromatographic column[11] with DNA as the stationary phase. This was possible because of the large favorable enantioselectivity in binding to calf thymus DNA exhibited by what we now confirm to be the Λ isomer. We had previously, and erroneously, assumed that the Δ isomer was binding more strongly. We report the confirmation of the correct configuration of the enantiomers by synthesis of a ruthenium(II) complex with a chiral bis-bpy like ligand ((+)-chiragen[6])[12] and ppz (of Δ configuration), and circular dichroism examination of this [Ru(chiragen)ppz] (+2) complex. Reexamination of the original CD spectra of the previously reported enantiomers of $Ru(bpy)_2(ppz)(+2)$ from the point of view of the exciton theory of optical activity[13] confirms this as the correct assignment. We also have measured the binding constants of the individual isomers with calf thymus DNA as a function of sodium chloride concentration in the range 10 to 100 mM, as well as an analysis of the Na^+ dependence of the binding constants using polyelectrolyte theory.[14] Conclusions regarding the generality of proposed binding models for such complexes are drawn.

MATERIALS AND METHODS

Materials

Calf thymus DNA was purchased from Sigma Chemical Company and purified by successive centrifugation and ethanol precipitation, followed by redissolving

in Tris buffer. All concentrations for the DNA are given as base pair concentrations. The extinction coefficient for calf thymus DNA phosphate used was 6600 cm^{-1} M^{-1}. Fluorescence measurements were performed in solutions containing pH 7.2 Tris buffer (0.0050M) with varying amounts of sodium chloride (0.010 to 0.10 M). All solutions were air saturated.

Ruthenium complexes were synthesized as previously described[1,10] and purified by column chromatography. Enantiomeric resolution of the $Ru(bpy)_2(ppz)(+2)$ ions was performed on a DNA/hydroxylapatite column as described.[11] The complex Ru((+)-chiragen[6])(ppz)(+2) was prepared from the Ru((+)-chiragen[6])Cl_2, which was characterized by absorption, NMR and CD measurements,[12] by reacting with ppz ligand according to published procedures. After column chromatography on alumina, the product showed an NMR spectrum, CD spectrum and UV-visible spectrum consistent with the formula above.

Instrumentation

UV-visible spectra were recorded using a HP8452 diode array spectrophotometer. Circular dichroism measurements were obtained on a Jasco 500C CD/ORD instrument. Fluorescence measurements were made using a Perkin-Elmer MPF-66 spectrometer interfaced to a Perkin-Elmer Model 7000 computer. Reported areas were obtained *via* integration of the fluorescence bands using the Perkin-Elmer Computerized Luminescence Software. NMR spectra were obtained on an IBM Brucker Model 200WP FT NMR spectrometer.

Fluorescence Titrations

Titrations were performed by adding aliquots of a solution containing a large excess of calf thymus DNA plus ruthenium(II) complex ion to a solution containing only ruthenium(II) complex ion at the same concentration (typically 6.1×10^{-6}M). Both solutions contained the same concentration of sodium chloride and Tris buffer. Integrated areas were used in the Eadie-Hofstee plots,[15–17] which were analyzed and plotted using Kaleidagraph software on a Macintosh computer. The quantity ΔF was obtained by subtracting the fluorescence intensity for the ruthenium complex ion solution only, from that of each solution with a known concentration of DNA base pairs. The plots of $\Delta F/[bp]$ *vs.* ΔF gave linear plots with R values greater than 0.996 for all data involving the $Ru(bpy)_2(ppz)(+2)$ enantiomers.

RESULTS

Assignment of Configuration

Synthesis and purification of the complex ion Ru((+)-chiragen[6])(ppz)(+2), which adopts predominantly the Δ configuration because of the rigid, chiral nature of the (+)-chiragen[6] ligand, allows us to assign the absolute configuration of the enantiomer of $Ru(bpy)_2(ppz)(+2)$ which binds more strongly to calf thymus DNA. The circular dichroism spectrum (Figure 1) of the Ru((+)-chiragen [6])(ppz)(+2) shows a negative peak at 303 nm ($\Delta\varepsilon = -135$) and a positive peak at 283 nm ($\Delta\varepsilon = +81$). The enantiomer of $Ru(bpy)_2(ppz)(+2)$ which shows[11] a negative peak ($\Delta\varepsilon = -130$) at 289 nm and a positive peak ($\Delta\varepsilon = +40$) at 273 nm is therefore assigned the Δ configuration. It is notable that the ultraviolet absorbance due to chelated bipyridine is shifted about 12 nm to the red in the (+)-chiragen[6] complexes as compared to the bis-bpy complexes. This is in accord with the red shift in the CD peaks. The Δ isomer is the one which is found in the dialysate when racemic $Ru(bpy)_2(ppz)(+2)$ is dialysed against calf thymus DNA, and which shows[1] a smaller hypochromic effect when binding to DNA as well as a smaller emission increase upon binding. The exciton theory of circular dichroism[13] predicts the same band structure for a $Ru(bpy)_2L(+2)$ complex in the vicinity of the most intense ultraviolet region bpy bands, *i.e.*, negative at the longer wavelength and positive at the shorter wavelength for the Δ enantiomer.

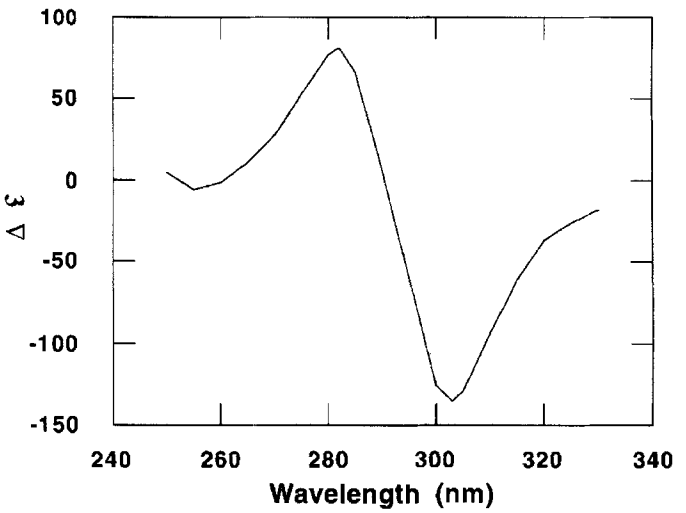


FIGURE 1 Circular dichroism spectrum of 1.0×10^{-5} 8M Ru((+)–chiragen[6])ppz(+2) in water.

Binding Constants

Eadie-Hofstee plots are shown in Figures 2 and 3 for binding of the Λ and Δ enantiomers of $Ru(bpy)_2(ppz)(+2)$, respectively, to calf thymus DNA. The sodium chloride concentrations used were 0.010, 0.025, 0.050 and 0.10 M. The graphical presentations illustrate the dependence of the K_b values on the sodium ion. Since K_b values are determined as −1/slope, in each case as the salt concentration increases from 0.010 to 0.10 M one can see progressive increase in the slope indicating a decrease in binding constant. The binding constant values are summarized in Table I. For the Λ enantiomer, the K_b values range from 3.9×10^3 (0.10 M NaCl) to 6.3×10^4 (0.010 M NaCl); for the Δ enantiomer they range from 1.2×10^3 (0.10 M NaCl) to 2.1×10^4 (0.010M NaCl). Binding to the Λ configuration over the Δ configuration is favored by 4.1, on average. The same procedure was carried out for Ru((+)-chiragen[6])(ppz)(+2), which has a Δ configuration. From the Eadie-Hofstee plots shown in Figure 4, the extracted binding constants (Table 1) indicate similarity in binding between the bis-bpy and (+)–chiragen[6] complex with a Δ configuration. For the (+)–chiragen[6] complex the K_b values vary from 1.3×10^3 (0.010 M NaCl) to 1.5×10^4 (0.10 M NaCl), indicating that both the absolute values and the sodium ion concentration dependence are quite similar to Δ-$Ru(bpy)_2(ppz)(+2)$.

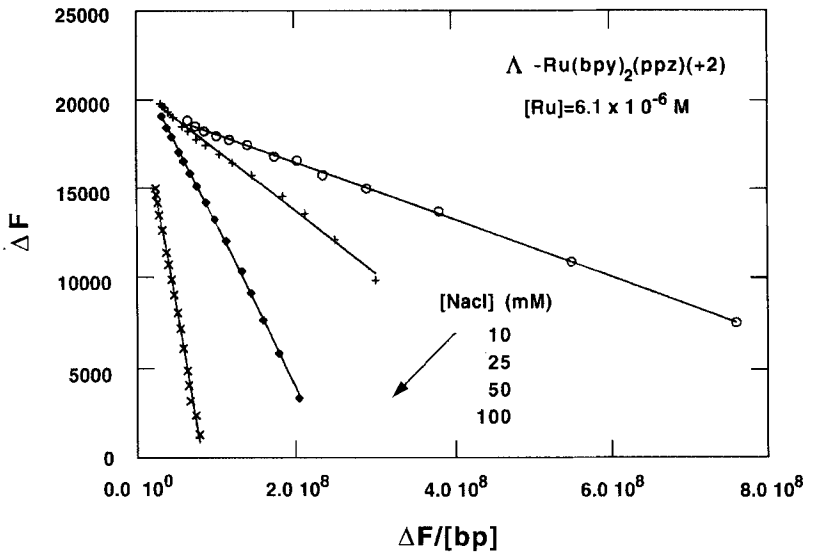


FIGURE 2 Eadie-Hofstee plots for binding of for Λ–$Ru(bpy)_2ppz(+2)$ with calf thymus DNA. Sodium chloride concentrations: 10mM (o), 25 mM (+), 50 mM (•), 100 mM (x).

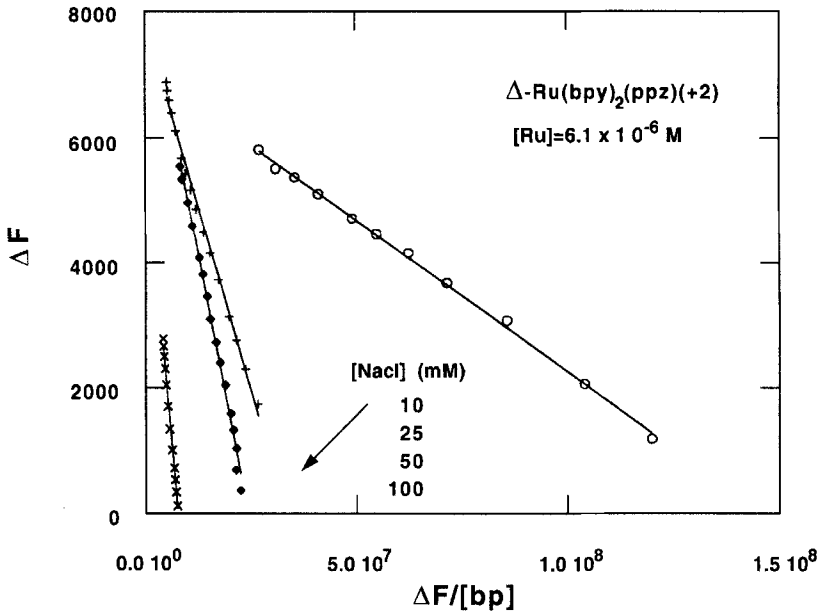


FIGURE 3 Eadie-Hofstee plots for binding of for Δ–Ru(bpy)$_2$ppz(+2) with calf thymus DNA. Sodium chloride concentrations: 10mM (o), 25 mM (+), 50 mM (•), 100 mM (x).

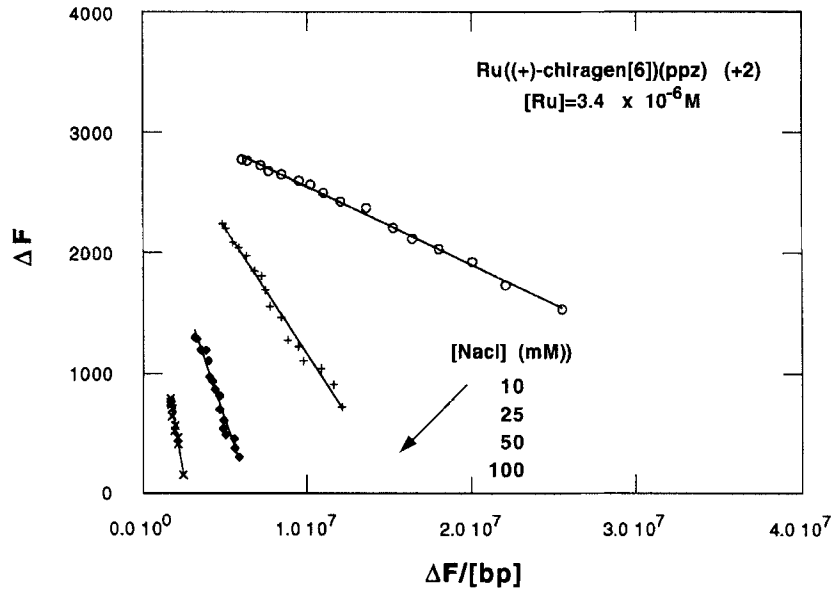


FIGURE 4 Eadie-Hofstee plots for binding of for Ru((+)–chiragen[6])ppz(+2) with calf thymus DNA. Sodium chloride concentrations: 10mM (o), 25 mM (+), 50 mM (•), 100 mM (x).

TABLE I Binding Constants for Calf Thymus DNA[a]

	Λ-Ru(bpy)$_2$(ppz) (+2)		*Δ-Ru(bpy)$_2$(ppz) (+2)*		*Ru((+)chiragen[6])(ppz)(+2)*	
[NaCl](M)	K_b *(X10^{-3})*	$K°_t$ *(% K_b)*	K_b *(X10^{-3})*	$K°_t$ *(% K_b)*	K_b *(X10^{-3})*	$K°_t$ *(% K_b)*
0.010	63	0.29 (0.47)	21	0.10 (0.50)	15	0.14 (0.93)
0.025	29	0.42 (1.4)	4.2	0.06 (1.5)	4.8	0.12 (2.5)
0.050	11	0.37 (3.4)	2.8	0.10 (3.6)	2.5	0.13 (5.3)
0.10	3.9	0.31 (8.0)	1.2	0.10 (8.3)	1.3	0.14 (11)

[a]K_b is the binding constant per base pair. $K°_t$ is calculated from equation (1) with the values of Z as follows: 1.38 (Λ-ppz); 1.36 (Δ-ppz); 1.20 ((+) chiragen[6]) ppz). All values for 25°C. It represents the non-electrostatic contribution to the overall binding constant.

Application of polyelectrolyte theory[14] to the binding of these complex ions to DNA is illustrated by Figure 5 and the calculated values in Table I based on equation (1) of reference 13:

$$\ln K_{obs} = \ln K^{o}_{t} + Z\xi^{-1} (\ln(\gamma\pm \delta) + Z\psi (\ln[M^{+}]) \quad (1)$$

where K^{o}_{t} represents the extent to which nonelectrostatic forces contribute to the overall binding constant and $Z\psi$ is the negative of the slope of a graph of ln K_{obs} *vs.* ln $[M^+]$. For double stranded B-form DNA, $\psi = 0.88$, $\delta = 0.56$ and $\xi = 4.2$; and $\gamma_{\pm}$ is the mean activity coefficient at cation concentration M^+.

At 0.050 M sodium chloride, the K^{0}_{t} values represent 3.4 to 5.3 percent of the overall binding constant, a value similar to that found for the enantiomers of Ru(phen)$_3$(+2). Here too the nonelectrostatic contribution to binding is small and the binding is largely electrostatic in origin.

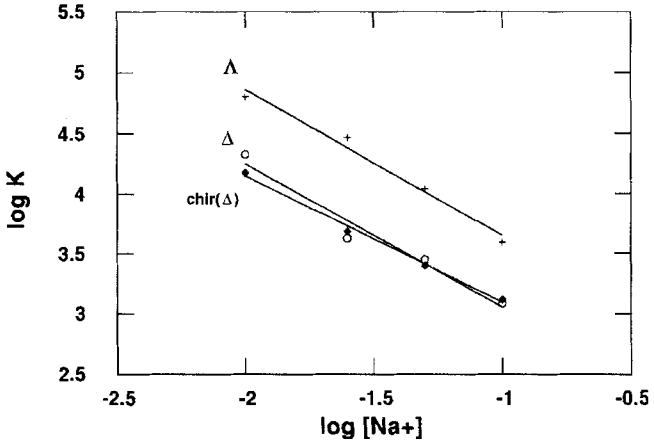


FIGURE 5 Log K *vs.* log $[Na^+]$ for Λ-Ru(bpy)$_2$ppz(+2) (+), Δ-Ru(bpy)$_2$ppz(+2) (o) and Ru((+)-chiragen[6])ppz(+2) (/DM).

DISCUSSION

Although counterexamples have been reported in the chemical literature, an ongoing assumption[1-2] regarding enantioselectivity of binding of intercalative tris-diimine complexes of metals such as ruthenium(II) and rhodium(III) with polynucleotides has been that the Δ enantiomer generally binds more strongly. The case has been most strongly made for complexes of the form, $Ru(phen)_2L$ (+2), and general applicability to similar complexes seems to be implied. Preferred binding of the Δ enantiomer is indeed found to be the case for a number of complexes and has been rationalized[7-8] based largely on viewing of molecular models and/or three-dimensional structures which appear to show more unfavorable van der Waals interactions between the two ancilliary (non-intercalative) ligands of the Λ enantiomer and the right handed structure of the major groove of DNA. The Δ enantiomer appears to more readily match the chirality of, and appears predisposed to nestle within, the major groove of B-form double helical DNA.

The finding for enantiomers of $Ru(bpy)_2(ppz)(+2)$ reported here is, of course, quite contrary to this relatively simplistic but, until now, seemingly convincing picture. For this complex, the Λ enantiomer in fact is found to bind more strongly to DNA by an average factor of about four-fold at sodium chloride concentrations ranging from 0.010 to 0.10 M. The overall driving force for binding is nevertheless found to be mostly electrostatic, as has been reported for $Ru(phen)_3(+2)$.

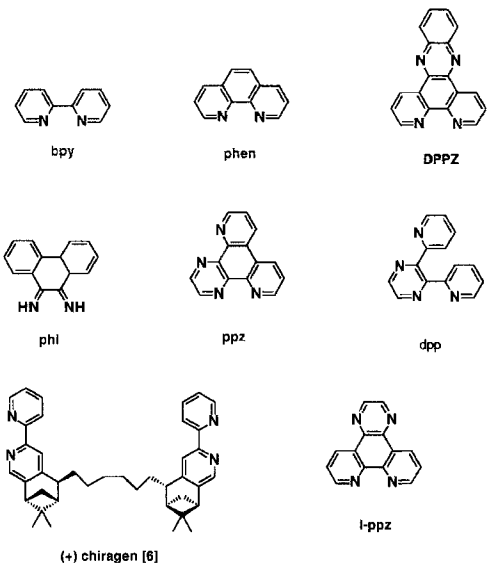
The further finding that the binding constants for Ru((+)chiragen[6])(ppz)(+2) are quite similar to the values for Δ-$Ru(bpy)_2(ppz)(+2)$ indicates the relatively small influence of the additional structure linking the two ancilliary bipyridine chelates. There is, however, a small difference in the slopes of the log K *vs.* log $[Na^+]$ plots indicating a slightly smaller influence of salt concentration on the binding of the(+)chiragen[6] complex. Note also that the non-electrostatic contribution to K_b is typically about twice as large for this complex. Indeed, the maximum fluorescence increase upon binding to DNA for these two complexes is quite similar, and about three-fold less than observed for the Λ enantiomer. This is consistent with a similar degree of penetration into the hydrophobic major groove for the two complexes of Δ configuration.

It is notable that for $Ru(bpy)_2(phi)(+2)$ examples in which a strong binding preference for the Λ enantiomer with two different polynucleotides have been reported[5], although a slight preference for the Δ enantiomer was found with calf thymus DNA. For complexes with two phenanthrolines, only $Ru(phen)_2(phi)(+2)$ binding to B-$p(dG\text{-}m^5dC)_2$ shows a marked preference for the Λ enantiomer. Whenever phen is involved as an ancilliary ligand, the interpretation is further complicated because coordinated phen can compete for the intercalative interaction

with other diimine ligands. This is not the case with coordinated bpy, however, as it shows no intercalating tendencies.

Interestingly, energy minimization calculations[9] for $Ru(phen)_3(+2)$ enantiomers binding to a duplex decamer of DNA indicate that a binding mode in which one phen is partially inserted between base pairs within the major groove is more favored for the Λ enantiomer (than for the Δ) over a binding mode in which the two ancilliary phen's lie deeper within the major groove with the third phen facing out. However, for both enantiomers, the partial intercalated mode is favored. The subtlety of the forces involved is exemplified by the partly inserted structures, in which the inserted phen does not lie parallel to the nearest base pairs of the double helix.

Possible interpretation of the structural basis for enantiomeric binding preference is further complicated by results of equilibrium dialysis experiments with calf thymus DNA and racemic $Ru(bpy)_2(i\text{-}ppz)(+2)$ (i-ppz: isomeric ppz; see structures) performed in our laboratory. Circular dichroism analysis of the dialysate indicates that binding of the Δ enantiomer is favored for this complex ion, where the only structural difference from the ppz complex is the change of position for a single ring nitrogen on the intercalative i-ppz ligand. Clearly, the subtle interplay of forces at work here renders the predictibility of specific enantiomeric preference in binding of such complexes to polynucleotides quite low at present. Further studies focusing on the binding differences between enantiomeric pairs of tris-diimine complexes of ruthenium(II) and related metals with DNA are required to increase understanding of this phenomonon.



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