Tris(phenanthroline)ruthenium(II) Enantiomer Interactions with DNA: Mode and Specificity of Binding†

S. Satyanarayana,‡ James C. Dabrowiak,§ and Jonathan B. Chaires*

Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505

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ABSTRACT: Absorbance and fluorescence methods, circular dichroism, UV melting experiments, viscosity, and competition dialysis were used to study the interaction of Δ and Λ tris(phenanthroline)ruthenium(II) with DNA. The results of these studies indicated that both isomers bind to DNA by a single mode. The two isomers differ, however, in their effect on the hydrodynamic properties of DNA as measured by viscosity and, therefore, probably differ in their individual binding modes. The optical properties of the fully bound compounds differ from those of the free, but the perturbations of their visible absorbance and fluorescence emission spectra are modest when compared to changes observed for other DNA binding compounds. Binding of both isomers to DNA was found to be weak (in comparison to proven intercalators), with binding constants on the order of 10^4 M^{-1} determined for their binding to calf thymus DNA. A small, positive *enthalpy* was found for the binding of each isomer to DNA, suggesting that binding is *entropically* driven. Both isomers increased the melting temperature of DNA, with little quantitative difference between the two. A modest base specificity was found for each isomer, with the Δ isomer preferentially binding to GC base pairs, and the Λ isomer preferentially binding to AT base pairs. Competition dialysis was used to examine the preference of Δ and Λ Ru for right-handed B DNA and left-handed Z DNA. Neither isomer exhibits significant selectivity for these radically different DNA secondary structures.

The use of metal complexes as probes of DNA structure and conformation is an active area of research (Tullius, 1989; Chow & Barton, 1992). Especially interesting are the metal complexes of the polypyridyl ligands, 2,2′-bipyridyl (bipy) and *o*-phenanthroline (phen), which exist as chiral molecules possibly capable of enantioselective recognition of DNA. Realizing the potential of these complexes as possibly useful probes of DNA secondary structure, Barton and co-workers (Barton, 1986; Fleisher et al., 1988) investigated the DNA binding and cleavage properties of a variety of metallopolypyridyls. Despite considerable effort by her group as well as by others, the binding mechanism of these compounds, a point which is central to understanding how they recognize DNA, remains controversial. The most well-studied compound of the group is $Ru(phen)_3^{2+}$ (Figure 1). The following is a brief review of what is known concerning the DNA binding mode of this compound and related polypyridyl analogs.

In 1976, Norden and Tjerneld used linear dichroism (LD) and circular dichroism (CD) to show that the chiral metal complex $Fe(bipy)_3^{2+}$ bound to calf thymus DNA in an enantioselective manner. Since it is known that this complex slowly racemizes in solution, the CD observed in the absorption bands of the compound was due to a shift in the enantiomeric equilibrium in the presence of DNA. This phenomenon, termed the Pfeiffer Effect (Pfeiffer & Quehl, 1931; Pfeiffer & Nakatsuka, 1933), is caused by inversion of a labile enantiomeric coordination compound while it is bound to an optically active substrate, in this case DNA. Although the details of the binding mechanism were not presented, Norden and Tjerneld (1976) suggested that intercalation was possible and that Δ-$Fe(bipy)_3^{2+}$ "yields the best fitting" on a right-handed DNA helix.

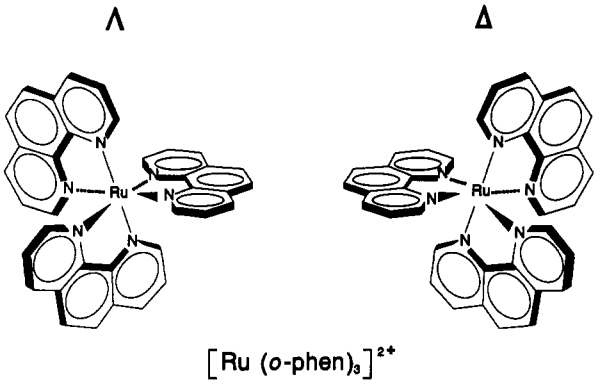

FIGURE 1: Structures of Δ and Λ Ru.

The pioneering work of Barton and co-workers began by studying the DNA enantioselectivity of tris(phenanthroline)-zinc(II)[1] (Barton et al., 1982), which, like $Fe(bipy)_3^{2+}$, is also a candidate for the Pfeiffer Effect (Kirshner et al., 1968). From unwinding assays with PM2 DNA and CD experiments involving dialysis, it was concluded that Δ-$Zn(phen)_3^{2+}$ preferentially bound to right-handed DNA by intercalation. Prompted by the observations of Barton et al. (1982) on the binding of the zinc complex to DNA, Yamagishi (1983) studied $Ru(phen)_3^{2+}$, which is stable and which cannot undergo rearrangements in the presence of DNA. Using electric dichroism, Yamagishi was able to show that the C_3 axis of the optically active forms of the compound, Δ and Λ Ru, were

† Supported by National Cancer Institute Grant CA35635 (J.B.C.).
* Author to whom correspondence should be addressed.
‡ On leave from the Department of Chemistry, Osmania University, Hyderabad, India.
§ Permanent address: Department of Chemistry, Syracuse University, Syracuse, NY 13244-4100.

[1] The accepted abbreviation of tris(phenanthroline)ruthenium(II) is $[Ru(o\text{-phen})_3]^{2+}$. This will be further shortened to Δ, Λ, and *rac* Ru, to denote the optically active isomers and the racemate, respectively.

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oriented differently relative to the DNA helix axis in the ruthenium–DNA complex. In a later work, Yamagishi (1984) used the angle measurements to conclude that Δ Ru is bound to DNA with one of its phenanthroline ligands intercalated between the base pairs while Λ Ru was not intercalated but was instead bound by an electrostatic mechanism.

In an extensive investigation involving unwinding studies using closed circular DNA and absorption and fluorescence spectroscopies, Barton and co-workers (Barton, 1983; Barton et al., 1984) argued that both Δ and Λ Ru intercalate between the base pairs of DNA. Both isomers unwound ColE1 DNA, leading to the conclusion that intercalation was an important binding mode. In comparison to other known intercalators, however, the hypochromic effect in the absorption spectra of bound Δ and Λ Ru is small. The modest change in optical properties upon binding was rationalized by suggesting that the electronic transition did not localize charge on the intercalated phenanthroline ring and that the polarization of the transition was in the wrong direction for a strong hypochromic effect. The increase in fluorescence lifetime of the bound compounds and the magnitude of their DNA binding constants were also used by Barton et al. (1984) as criteria for intercalation.

Using an assay involving the DNA unwinding enzyme topoisomerase I, Kelly et al. (1985) showed that *rac* Ru unwinds covalently closed circular pBR322 DNA, thus confirming the earlier unwinding studies with the optically active forms of the compound (Barton et al., 1984). Since *rac* Ru increased the melting point of poly[d(AT)] in a manner similar to that of ethidium bromide, it was suggested that the coordination compound bound to DNA by intercalation.

Barton et al. (1986) also studied the binding of Δ and Λ Ru to nucleic acids of different base composition and structure using equilibrium dialysis and photophysical methods. From measurements of the fluorescence emission lifetime of the bound isomers, it was concluded that Δ and Λ Ru used *both* an intercalative and surface binding mode in interacting with DNA. Nonlinear Stern–Volmer plots using ferrocyanide as a quenching agent for the bound Ruthenium compound were interpreted in terms of *two bound* species for each isomer, with *each* bound species having different quencher accessibilities.

The possibility that the ruthenium compounds could only partially insert one phenanthroline ring was first raised by Barton et al. (1984), and later by Kelly et al. (1985) and Gorner et al. (1988). It was argued (Gorner et al., 1988) that complete insertion, as occurs with acridinium and ethidium, was blocked by the two external phenanthroline rings which clash with the DNA backbone. Molecular modeling and energy minimization calculations (Haworth et al., 1991) for the most part agree with the proposal that partial insertion occurs. Calculations showed that each isomer has two binding modes, partial insertion (*not* classical intercalation) and external or electrostatic binding. Binding was proposed to occur in the major groove of DNA.

Norden and co-workers (Hiort et al., 1990) provided a another view of the ruthenium–DNA interaction. From linear dichroism measurements showing the orientation of the bound metal complex relative to the helical axis of DNA (Hiort et al., 1990) and from the small magnitude of spectroscopic changes upon binding, it was concluded that each isomer bound to DNA by a *single* binding mode and that *neither* isomer is bound to DNA by intercalation. This view was further supported by two-dimensional NMR measurements (Eriksson et al., 1992) which showed that both Δ and Λ Ru bind by a nonintercalative mode within the minor groove of DNA. Furthermore, for the duplex $[d(CGCGATCGCG)]_2$, all NMR NOE contacts are located around the AT sequence indicating that binding for either isomer takes place at the AT site in the center of the duplex. Rehmann and Barton (1990a,b) also used NMR to study the ruthenium–DNA interaction. For the oligonucleotides $[d(GTGCAC)]_2$ and $[d(CGCGCG)]_2$, they concluded that Δ Ru prefers intercalation while Λ Ru prefers surface binding. Although the location of the intercalative mode could not be deduced from their NMR data, surface binding appeared to occur in the minor groove as evidenced by chemical shift changes in adenine H2.

While the nature of the binding mode has been the focus of many investigations, other aspects of the ruthenium–DNA interaction have also been reported. Barton et al. (1984) showed that in 4 M NaCl the affinities of both isomers for the Z form of poly(dGdC) were the same. However, in view of the relatively low binding constants of the isomers, 10^3–10^4 M^{-1} (Hiort et al., 1990; Rehmann & Barton, 1990a; Barton et al., 1984), and the high concentration of salt in the medium, it is not clear if significant binding occurred under the conditions of the study to definitively evaluate the relative specificity of Ru isomers for B and Z DNA. Hard et al. (1987) also examined the enantioselectivity of the Ru isomers for right- and left-handed DNA. Equilibrium dialysis experiments with poly(dGm^{5}dC), a polymer which readily adopts the Z conformation under low salt conditions, showed that Δ Ru binds more tightly to both the B and Z forms of poly(dGm^{5}dC) than does Λ Ru. Binding of both isomers appeared to induce an allosteric conversion of poly(dGm^{5}dC) from the Z to the B form of the polymer, resulting in cooperative binding of the isomers. Because of the complexity of the binding isotherms in these cases, quantitative evaluation of the relative affinity of Ru isomers for B and Z DNA was made difficult.

The potential of tris(phenanthroline)ruthenium isomers as probes of DNA secondary structure and the controversy surrounding their binding mechanism led us to reinvestigate their interactions with DNA. We considered it important to determine the number and types of binding modes utilized by each isomer. With this information, it would be possible to identify what features of DNA are being recognized, which is essential for further development of the compounds as practical and sensitive probes of DNA structure. Additional thermodynamic information is needed to determine how useful the ruthenium isomers are as "probes" of DNA structure. We have a long-standing interest in the quantitative evaluation of both site- and sequence-specific ligand binding to DNA (Chaires, 1992; Dabrowiak et al., 1992; Goodisman & Dabrowiak, 1992) and in the quantitative evaluation of the conformational specificity in DNA ligand binding (Chaires, 1983, 1985, 1986a,b; Herrera & Chaires, 1989; Gilbert et al., 1991a,b). The potential of novel specificity in the interaction of Δ and Λ Ru with DNA further motivated the current studies.

In a preliminary report (Satyanarayana et al., 1992), we provided evidence that each isomer has only a single binding mode and that neither Δ nor Λ Ru binds to DNA by classical intercalation. The salt dependency of their DNA binding constants indicated that the interaction of Δ and Λ Ru with DNA is largely electrostatic in nature and thus is unlike other known DNA intercalating agents.

In this work, we further characterize the ruthenium–DNA interaction. We present the optical properties of the fully bound ruthenium isomers and use data from fluorescence titration experiments to determine binding constants for the compounds. Analysis of steady-state fluorescence data using

the method of singular value decomposition (SVD) is presented to prove that the binding of both isomers to DNA involves only two states, free and bound, thus reinforcing the proposal that each isomer has only a *single* binding mode. We also show that the quenching agent $K_4[Fe(CN)_6]$ cannot be used to differentiate multiple modes of bound ruthenium as earlier claimed (Barton et al., 1986). Optical difference spectra as a function of temperature and DNA melting studies are described that show that the binding enthalpy for the Ru–DNA interaction is a small positive value and that isomer binding to DNA is therefore entropically driven. Finally, competition equilibrium dialysis experiments are described that show the base specificity of the isomer binding to DNA and which indicate that neither isomer binds preferentially to Z DNA. On the basis of these new experimental findings, the potential of tris(phenanthroline)ruthenium as a useful probe of DNA structure may be discussed.

MATERIALS AND METHODS

Materials. The synthesis and resolution of the ruthenium compounds as their perchlorate salt was as earlier described (Dwyer & Gyarfas, 1949; Dwyer et al., 1947). The concentrations of metal complexes were determined from absorbance measurements at 447 nm using the molar extinction coefficient $\epsilon_{447} = 19\,000\ M^{-1}\ cm^{-1}$ (Lin et al., 1976). Calf thymus DNA was purchased from Sigma Chemical Co. (lot no. 42F 9555) or Boehringer Mannheim, Inc., Biochemical Division (lot no. 1154022323) and was sonicated and purified as previously described (Chaires et al., 1982). The DNA was dialyzed two times in the required buffer before use. *Clostridium perfringens* DNA and *Micrococcus lysodeikticus* DNA were purchased from Sigma Chemical Co. (St. Louis, MO).

Buffers. Experiments were carried out in the following buffers: buffer A (BPE), 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0; buffer B, 5 mM Tris-HCl, 50 mM NaCl, pH 7.1; buffer C, 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM Na_2EDTA, pH 7.0; buffer D, 5 mM Tris-HCl, 10 mM NaCl, pH 7.1.

Instruments. Absorbance spectra were recorded using a Cary Varian 219 UV–visible spectrophotometer with 1-cm quartz cells. DNA melting experiments employed a Perkin-Elmer lambda 3 UV–visible spectrophotometer using stoppered 1-cm quartz cuvettes. Fluorescence data were recorded with a Perkin-Elmer 650-40 fluorescence spectrophotometer. Circular dichroism (CD) spectra were recorded using a Jasco Instruments J500A spectropolarimeter in a 1-cm path length cell.

Fluorescence Measurements. Fluorescence quenching experiments were carried out at 20 °C by the addition of microliter aliquots of 0.02 M $K_4[Fe(CN)_6]$ to 2-mL samples of Δ or Λ Ru in buffer B. For the quenching experiments, samples were excited at 455 nm and emission was monitored by collecting all emitted light passing through a 495-nm-cutoff filter. In experiments where ionic strength was maintained, KCl was added along with $K_4[Fe(CN)_6]$ such that the final, total concentration of K^+ was constant at 4×10^{-3} M. Data were transformed to construct a Stern–Volmer plot according to the expression $F_0/F = 1 + K_{SV}[K_4Fe(CN)_6]$, where F_0 is the intensity of fluorescence in the absence of quencher, F is the fluorescence intensity in the presence of quencher, and K_{SV} is the Stern–Volmer quenching constant. Quenching curves were analyzed by linear and nonlinear least-squares methods using the software package FitAll, v. 5, (MTR Software, Toronto). Data from nonlinear Stern–Volmer plots were fit, after transformation, to the equation

$$\frac{F}{F_0} = \sum_{i=1}^{n} \frac{f_i}{1 + {}_iK_{SV}[Q]}$$

to extract K_{SV} values and to decide on the number (i) and fractional amount (f_i) of species present by a statistically rigorous method (Eftink, 1991).

Thermal Melting Experiments. These experiments were conducted in buffer C using continuous heating from 20 to 100 °C at a rate of 1 °C/min. Absorbance increases at 270 nm were measured relative to an unheated reference cell containing a ruthenium concentration identical to that in the heated sample cell. DNA concentrations were constant in these experiments at 48 μM bp, while the concentration of Ru isomers was varied from 3.8 to 18.8 μM. The fraction of single strands was estimate from the relation $f_{ss} = (A - A_0)/(A_f A_0)$, where A_f and A_0 are the final and initial absorbance values at 270 nm, respectively, and A is the observed absorbance at a given temperature. This normalization procedure neglects possible contributions to the absorbance change at 270 nm from the dissociation of the ruthenium compounds over the course of the melting transition. We expect that such contribution will be small, since absorbance changes were monitored relative to a reference cell containing appropriate concentrations of Δ or Λ Ru, as described above.

Thermal Difference Spectra. These experiments were carried out with identical concentrations of ruthenium and DNA in both the reference and sample cells of the spectrophotometer. Temperatures in both compartments were maintained to ±0.1 °C using a Fischer Isotemp circulator (Model 900). Keeping the reference temperature at 25 °C, difference spectra at sample temperatures of 9 and 45 °C were recorded.

Competition Dialysis. Competition dialysis, introduced by Mueller and Crothers (1974), is an elegantly simple and direct method for assessing preferential ligand binding to DNA samples of differing sequence or conformation. In the competition dialysis experiment, a three-chambered dialysis cell is used, in which two DNA samples at identical concentrations occupy two separate chambers that are separated from the third (containing ligand) by dialysis membranes. After equilibration, all chambers contain identical concentrations of free ligand, but the DNA containing chambers will have differing amounts of total ligand *if* there is preferential interaction of the ligand with one of the DNA samples. Our experiments were carried out in buffer D using a commercial dialysis device (Spectrum Medical Industries, Los Angeles) and Spectrapor dialysis tubing (MW cutoff 6000–8000). In separate experiments the free ruthenium concentration in the central chamber was varied over the range 1–20 μM. In experiments determining the base specificity of the compound, the DNA pair *C. perfringens* (31% GC) and *M. lysodeikticus* (71% GC) was used. DNA, at identical concentrations of 0.122 mM bp, was placed in the two outer compartments. After dialysis with continuous agitation for 48 h to reach equilibrium, the concentrations of free (C_f) and total (C_t) ruthenium in the dialysate and retentate, respectively, were determined by absorbance at the isosbestic point using $\epsilon_{464} = 13\,600\ M^{-1}\ cm^{-1}$. The amount of bound drug (C_b) was determined from the difference, $C_t - C_f = C_b$.

The ligand specificity for DNA secondary structure was determined in buffer D containing 5 mM $MgCl_2$ and ruthenium concentrations in the range 2–25 μM. The polymers poly(dGm^{5}dC) and poly(dGdC) were used at concentrations of 100 μM and were in separate dialysis chambers. Under

these conditions, poly(dGm^{5}dC) adopts the Z conformation, while poly(dGdC) remains in the B conformation. After equilibrium is reached, the concentrations of ruthenium in the dialysate and retentate were determined using absorption spectroscopy as described above. As a control, the experiments were repeated in buffer D with no added $MgCl_2$, conditions in which both polymers adopt the B-form conformation.

Singular Value Decomposition. Singular value decomposition (SVD) is a powerful, model free, analytical tool that allows for the enumeration of the significant species contributing to a family of experimental spectra (Henry & Hofrichter, 1992; Johnson, 1992). Briefly, SVD decomposes a data matrix **A**, constructed from a series of experimental spectra, into the product of three matrices, $\mathbf{A} = \mathbf{USV}^t$. The **S** matrix contains only diagonal elements, the so-called singular values. The number of significant singular values is equal to the number of significant spectral species within the experimental data. The product **US** yields the basis spectra, the spectra from which the entire family of experimental spectra may be calculated with the appropriate weighting factors (found in the **V** matrix). In our case, we titrated a fixed amount of Δ or Λ Ru (10 μM) with DNA (0–2 mM bp) and recorded complete fluorescence emission spectra after each addition. In this system, there will be at least two species, free and bound. If Ru is bound by more than one mode (i.e., intercalated *and* outside), and if these binding modes result in differing fluorescence emission properties, then SVD should indicate more than two significant singular values. SVD was performed using routines available in PCMATLAB (The Mathworks, Inc., Natick, MA). Because of limitations on the size of matrices that can be manipulated on personal computers, analysis was done using a data matrix **A** constructed from fluorescence emission spectra spanning 540–690 nm, with data spaced at 5-nm intervals. Spectra obtained at 12 DNA concentrations were included. The number of significant singular values was determined using the criteria set forth by Henry and Hofrichter (1992). These included (i) the magnitudes of the singular values, (ii) first-order autocorrelation values of the columns of **U** and **V**, and (iii) the ability to quantitatively reconstruct the data matrix **A** after elimination of singular values believed to be insignificant.

Viscosity Studies. Viscosity experiments used an Ostwald-type viscometer, immersed in a thermostated water bath maintained at 27 (±0.1) °C. Titrations were conducted without removing the DNA samples from the viscometer, by using a Stoelting, Co. Model 51218M microsyringe assembly to add ligand, followed by bubbling with nitrogen to ensure mixing. The amount of bound ligand was determined by parallel optical titration experiments, using identical sample and titrant volumes and concentrations. DNA samples approximately 200 bp in length were prepared by sonication (Chaires et al., 1982) for these studies, in order to minimize complexities arising from DNA flexibility. For the viscometer used, a buffer flow time, t^0, of 76.34 (±0.06) s was observed. Relative viscosities for DNA in the presence and absence of ligand were calculated (neglecting possible changes in axial ratios) from the relation $\eta = (t - t^0)/t^0$, where t is the observed flow time. A DNA concentration range of 1.0–2.8 mM bp was investigated, concentrations that yielded flow times of ≥90 s. Data are presented as plots of L/L_0 versus r bound, according to the theory of Cohen and Eisenberg (1969). L/L_0 is calculated directly from the experimental viscosity data and is the ratio of contour lengths in the presence (L) and absence (L_0) of bound ligand, under conditions where the DNA has been prepared such that its size is below the persistence length, and it therefore behaves as a rod-like macromolecule.

Table I: Summary of Optical Properties of Free and Bound Ruthenium Compounds[a]

	Δ Ru	Λ Ru
absorbance		
λ_{max}(free)	447	447
λ_{max}(bound)	432	426
λ_{iso}[b]	464	464
ϵ_f (at λ_{max})	19 000	19 000
ϵ_b (at λ_{max})	15 660	16 300
fluorescence		
λ_{max}(ex)	455	455
λ_{max}(em)	592	592
F_b/F_0	2.25	2.05
circular dichroism		
λ, Δε(free)	463, −22.2	463, +20.3
	418, +15.2	418, −13.7
λ, Δε(bound)	470, −19.0	469, +27.6
	420, +15.4	423, −8.7

[a] Units: λ, nm; ε, M^{-1} cm^{-1}; $\Delta\epsilon = (\epsilon_l - \epsilon_r)$, M^{-1} cm^{-1}. [b] Wavelength at the isosbestic point.

RESULTS AND ANALYSIS

Changes in the optical properties of Δ and Λ Ru upon binding to DNA are summarized in Table I. (Primary data used to compile Table I are shown in Figures S1–S3 in the supplementary material.) Particular care was taken in these studies to add a large enough excess of DNA to ensure complete binding of the Ru isomers, so that the limiting free and bound spectra could be determined. Because of the low affinity of these compounds for DNA, molar ratios of DNA bp to compound of over 100 are required to ensure complete binding. Table I shows that the molar extinction of both isomers decreases upon binding to DNA at wavelengths near the absorbance maximum of the free compounds. An isosbestic point is observed for both isomers at 464 nm. The spectra of the bound isomers are slightly blue shifted relative to that observed for the free isomers in solution, although the magnitude of the shift is not large. Table I shows that the magnitudes of the CD spectra of both Δ and Λ Ru change slightly upon complexation with DNA, with changes of slightly larger magnitude apparent for the Λ isomer. Fluorescence emission spectra for the free and bound forms of the Ru isomers show that emission is enhanced for both Δ and Λ Ru. The ratio of the fluorescence intensity of bound to free compound at 592 nm (with λ_{ex} = 455 nm) is slightly greater for Δ Ru (F_b/F_0 = 2.25) than for Λ Ru (F_b/F_0 = 2.05) (Table I; Figure S3).

The method of singular value decomposition (SVD) (Henry & Hofrichter, 1992; Johnson, 1992) was used to assess the number of significant species contributing to the steady-state emission spectra of Δ and Λ Ru over the course of a titration of fixed amounts of compound with increasing amounts of DNA. SVD provides enumeration of the number of significant spectral species without reference to, or assumptions about, a particular binding model. Twelve emission spectra were obtained at 10 μM Ru with DNA varied over the range of 1–1000 μM bp. These spectra were used to construct the data matrix **A**, using a wavelength range of 540–690 nm, with data spaced at 5-nm intervals. SVD returned 12 singular values, the four highest of which are 80.96, 1.93, 0.43, and 0.28. Only the first two of these are statistically significant, and only two nonrandom basis spectra and amplitude vectors result from the analysis, as judged by the criteria set forth in Henry and Hofrichter (1992). (Primary fluorescence data,

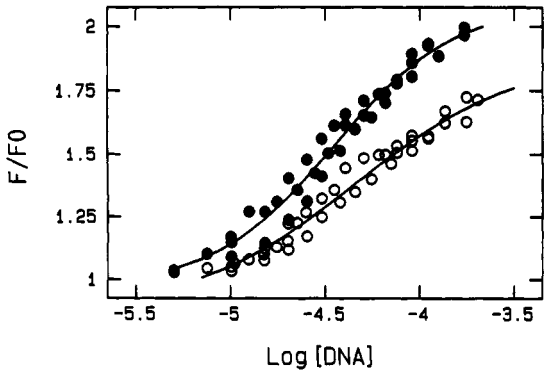

FIGURE 2: Fluorescence titration of calf thymus DNA with Δ Ru (filled circles) and Λ Ru (open circles). Total Ru concentration was held constant during titration with increasing amounts of DNA. The quantity F/F_0 is the ratio of total fluorescence emission in the presence (F) and absence (F_0) of DNA.

computed basis spectra, and amplitude vectors from SVD analysis are presented in the supplementary materials.) These results conclusively indicate that only two spectral components are required to account for the changes in the fluorescence emission spectra over the course of titration with DNA. Binding to DNA is therefore strictly a two-state process. Further, the results of SVD strongly suggest that there is only a single binding mode. If multiple binding modes do exist, their steady-state fluorescence emission properties must be identical, an unlikely possibility if intercalation and surface (or groove) binding are considered as two possible binding modes, since these two modes would place the fluorophore into radically differing environments.

Figure 2 shows the results from fluorescence titration experiments in which fixed concentrations of Δ and Λ Ru were titrated with DNA. Data were obtained at total Δ or Λ Ru concentrations of 1, 5, and 10 μM. The observed titration midpoints were independent of total ruthenium concentration over that range, an indication that the dissociation constant for the interaction of these compounds with DNA is greater than 10 μM. The curves shown in Figure 2 span several logarithmic units in DNA concentration, as expected from the general properties of titration curves. Figure 2 illustrates the large molar excess of DNA required to fully bind all of the ligand. The maximum fluorescence change upon DNA binding for each compound was determined independently by measuring their fluorescence emission spectra in the presence of a 4000-fold molar excess of DNA base pairs (see Figure S3, Table II). By assuming two states for the ligand (free and bound), and the values of F_b/F_0 listed in Table II, the data of Figure 2 may be quantitatively analyzed to yield binding constants (K) and neighbor exclusion values (n) for the neighbor-exclusion model of McGhee and von Hippel (1974). For Δ Ru, values of $K = 4.9(\pm0.3) \times 10^4\ M^{-1}$ and $n = 3.7$ (±0.2) were obtained. Corresponding values for the Λ isomer were found to be $K = 2.8(\pm0.2) \times 10^4\ M^{-1}$ and $n = 3.4$ (±0.3). The Δ isomer thus binds slightly more tightly to DNA than does the Λ isomer, a fact that is evident in the primary data shown in Figure 2 by the differences in the titration midpoints observed for the two compounds. A more thorough discussion of the binding data has been presented elsewhere (Satyanarayana et al., 1992).

Fluorescence quenching studies using $K_4[Fe(CN)_6]$ as an anionic quencher were conducted to further probe the possible multiplicity of binding modes of Δ and Λ Ru (Figures 3 and 4). These studies partially repeat published studies (Barton et al., 1986) that we believe neglected two critical factors in

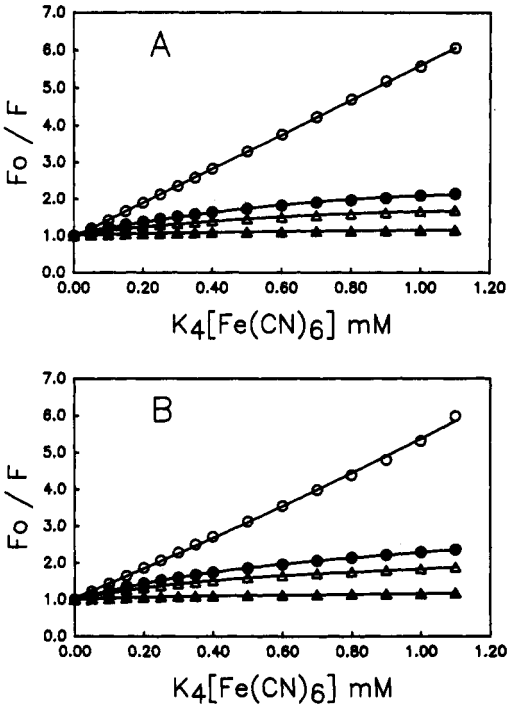

FIGURE 3: Fluorescence quenching, F_0/F of Δ Ru (A) and Λ Ru (B) with increasing concentrations of $K_4[Fe(CN)_6]$, in buffer B. The ionic strength of the solution was not maintained upon addition of quenching agent. Open circles refer to ruthenium alone (2 μM); other symbols refer to ruthenium (2 μM) plus DNA at total compound to base pair ratios (r) of (filled circles) $r = 0.05$; (open triangles) $r = 0.025$; and (filled triangles) $r = 0.002$.

Table II: Comparison[a] of Selected Physical Properties for Compounds of Known DNA Binding Modes with Those of Δ and Λ Ru

property	ethidium	daunomycin	Hoechst 33258	Δ/Λ Ru
binding mode	intercalator	intercalator	groove binder	?
known X-ray structure?	yes	yes	yes	no/no
optical changes upon binding				
absorbance				
λ_{max}(free)	479 nm	480 nm	338 nm	447/447 nm
$\Delta\lambda_{max}$	+38 nm	+25 nm	+22 nm	−15/−21 nm
ϵ_b/ϵ_f	0.44	0.61	0.59	0.82/0.86
fluorescence				
F_b/F_0	11–21	0.05	>50	2.25/2.05
τ_b/τ_f	12.5	0.6	?	5.0/5.0
accessible to [Q]?	no	no	no	no/no
effect on T_m	inc	inc	inc	inc/inc
effect on DNA viscosity	inc	inc	none	dec/none
DNA unwinding?	yes	yes	no	yes/yes

[a] Key: λ_{max}(free) is the absorbance maximum of the free compound; $\Delta\lambda_{max}$ is the shift in absorption maximum upon DNA binding. ϵ_b/ϵ_f is the ratio of molar extinction coefficients of the bound and free forms at λ_{max}(free). F_b/F_0 and τ_b/τ_f are the ratios of fluorescence emission intensity and the fluorescence lifetimes, respectively, of the bound and free forms of the ligand.

attempting to characterize the quenching of Δ and Λ Ru when bound to DNA, namely, the large excess of DNA required to completely bind the compounds and the necessity to maintain a constant ionic strength over the course of the titration with quencher. Figure 3 shows the results of quenching experiments, cast into the form of Stern–Volmer plots, for Δ and Λ Ru free in solution and in the presence of increasing amounts of DNA. In the absence of DNA, both compounds show linear Stern–Volmer plots, the slopes of which yield quenching

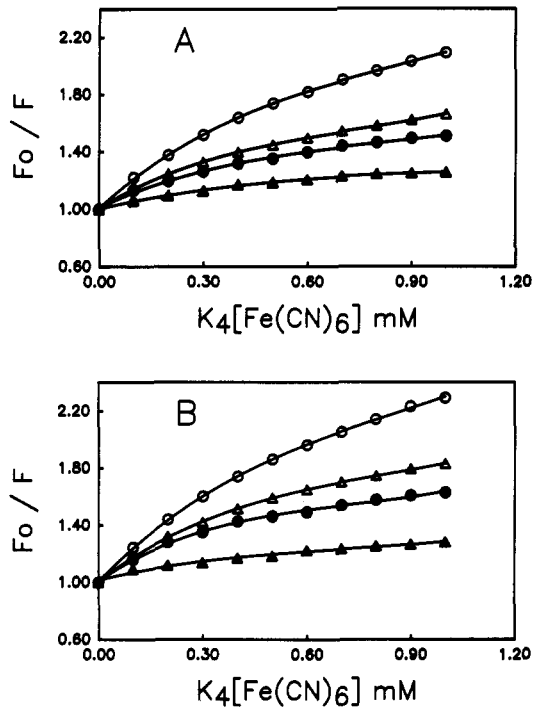

FIGURE 4: Fluorescence quenching, F_0/F, of (A) Δ Ru and (B) Λ Ru in the presence of calf thymus DNA with increasing concentrations of $K_4[Fe(CN)_6]$ in buffer B. The isomer concentration was constant at 4 μM. Open symbols refer to experiments in which ionic strength was not maintained constant. Solid symbols refer to experiments conducted at constant ionic strength. Circles (both open and solid) refer to DNA bp:Ru ratios of 0.05. Triangles (both open and solid) refer to DNA bp:Ru ratios of 0.025.

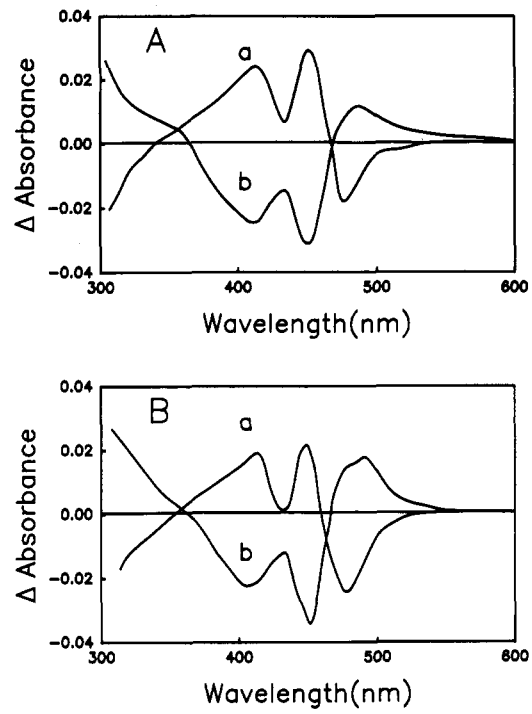

FIGURE 5: Thermal difference spectra of (A) Δ Ru and (B) Λ Ru (10 μM) bound to calf thymus DNA (20 μM) in buffer D. Spectra are the difference between reference at 25 °C and sample at 9 °C (a) or 45 °C (b).

constants of 4514(±42) M^{-1} and 4280(±25) M^{-1} for the Δ and Λ isomers, respectively. At a DNA (bp):Ru ratio of 20:1 and 40:1, Figure 3 shows apparently biphasic plots for both isomers. These data reproduce the quenching curves presented by Barton et al. (1986). At a ratio of 500:1, a DNA concentration excess sufficient to ensure complete ligand binding, the Stern–Volmer plots for both compounds have essentially zero slope, indicating that the bound species is inaccessible to added quencher. Figure 4 shows the titration of 20:1 and 40:1 solutions in more detail and illustrates the necessity for strictly maintaining a constant ionic strength over the course of the titration with quencher. If ionic strength is not maintained, there is a net dissociation of Ru from DNA, as expected from polyelectrolyte theory, resulting from the increase in monovalent cation concentration, and the fact that both Δ and Λ Ru carry a net charge of 2+. While such little quencher is being added, it might be supposed that the effect would be negligible, but because of the stoichiometry of $K_4[Fe(CN)_6]$, amounts of the cation are in fact being added in sufficient quantity to dissociate bound Ru from the DNA. The effect is clear in Figure 4. By not maintaining ionic strength, the ratio F_0/F increases much more sharply than in the cases where ionic strength is constant, and the curves are decidedly nonlinear. Both of these features might lead to erroneous interpretations about the number of species accessible to quencher. When ionic strength is maintained, quenching curves remain nonlinear, a result that we attribute to the fact that at these ratios of DNA:Ru, there is a considerable fraction of unbound compound. Nonlinear least-squares analysis of the 20:1, constant ionic strength, curves shown in Figure 4 supports that notion. For Δ Ru, the analysis shows that two components are sufficient to describe the curve, one with K_{SV} = 4931 M^{-1} contributing 31% of the initial fluorescence intensity, the second with K_{SV} = 139 M^{-1} contributing 69% of the initial fluorescence intensity. Note that K_{SV} for the first component is nearly identical to that observed for the free compound alone. For the Λ isomer, similar results are obtained from the analysis, with K_{SV} = 4942 M^{-1} (42%) and K_{SV} = 70.5 M^{-1} (58%) observed. These results indicate that when ionic strength is maintained, only two species are required to account for the quenching curves, corresponding to the free Ru and *one* bound form. The bound form is essentially inaccessible to added quencher. There is no evidence in these data to support the proposal that multiple bound forms exist with differing quencher accessibilities. The nonlinearity in Stern–Volmer quenching curves at DNA concentrations insufficient to fully complex ligand arises from the presence of a substantial fraction of free ligand, fully accessible to quencher.

Equilibrium dialysis experiments at temperatures of 5 and 37 °C were attempted to obtained binding isotherms that might provide estimates of the DNA binding enthalpy of Δ and Λ Ru (data not shown). The results of these experiments showed, for both isomers, changes in the binding constant of less than a factor of 2 over the temperature range studied, indicating a DNA binding enthalpy of nearly zero. Thermal difference spectroscopy was used to investigate the temperature dependence of Δ and Λ Ru binding to DNA with greater precision and sensitivity. The results are shown in Figure 5. For both Δ and Λ Ru, an increase in sample temperature from 25 to 45 °C results in a *decrease* in absorbance at λ_{max}, indicative of *increased* DNA binding. The effect is small, as seen by the scale of the ordinate in Figure 5. The shapes of the thermal difference spectra indicate that the absorbance changes are not a simple result from the thermal expansion of solvent. Both positive and negative peaks are evident, as are clear isosbestic points. Upon decreasing the temperature to 9 °C, there is an *increase* in absorbance, corresponding to *decreased* Ru binding. These results indicate that the DNA binding enthalpies of both Δ and Λ Ru are of positive sign. Further, the magnitude of the spectral changes observed,

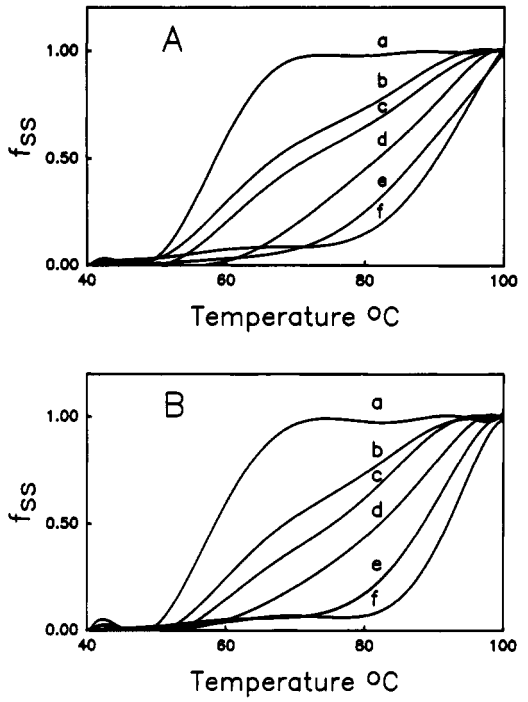

FIGURE 6: Thermal melting curves for calf thymus DNA in presence of (A) Δ Ru and (B) Λ Ru in buffer C. Curves: (a) DNA alone (48 μM); (b–f) increasing amounts of ruthenium. Amounts of Ru: (b) 3.8 μM, (c) 5.6 μM, (d) 9.4 μM, (e) 14.2 μM, (f) 18.8 μM. The quantity f_{ss} is the fraction of single strands in solution.

coupled with the results obtained by equilibrium dialysis, suggests that the magnitude of the enthalpy change is small. Both Δ and Λ thus bind to DNA with a small, but positive change in enthalpy. DNA binding must therefore be largely entropically driven.

Binding of both Δ and Λ Ru to DNA dramatically increases its melting temperature, as shown in Figure 6. At less than saturating amounts of added Ru, melting curves are seen to be biphasic, as expected from theoretical treatments of effects of ligand binding on the thermal denaturation of DNA (Crothers, 1971; McGhee, 1976). At higher Ru concentrations, small absorbance changes at low temperatures are evident, probably arising from thermally induced changes in the ligand binding ratios. From the data of Figure 6, quantitative information about the Ru–DNA interaction may be obtained. McGhee (1976) has shown that under conditions where the DNA helix is saturated with ligand, the shift in T_m is given by the relation

$$1/T_m^0 - 1/T_m = (R/\Delta H)(\ln [1 + KL])^{1/n} \qquad (1)$$

(assuming that there is no ligand binding to single-stranded DNA). The symbols in eq 1 are defined as follows: T_m^0 is the melting temperature of DNA in the absence of ligand, T_m is the melting temperature under saturating conditions, R is the gas constant, ΔH is the enthalpy of DNA melting (per base pair) under the conditions of the experiment, K is the ligand binding constant (at T_m), n is the exclusion parameter, and L is the free ligand concentration at T_m. For the calf thymus DNA used in these studies, under identical solution conditions, a melting enthalpy of 6.9 kcal/mol was determined by differential scanning calorimetry (J. B. Chaires, unpublished data). From the experimentally determined changes in T_m, K values may be estimated using eq 1 by assuming n values determined by equilibrium binding experiments at room temperature and by assuming that L is equal to the total ligand concentration. From such an analysis, a value of $K = 5.5 \times 10^4$ M^{-1} may be calculated for Δ Ru and $K = 6.7 \times 10^4$ M^{-1} may be calculated for Λ Ru, with both values referring to the temperature $T_m \approx 90$ °C. Note that these values are slightly higher than the binding constants determined by more traditional methods at room temperature, a finding consistent with our conclusion that the binding enthalpy is of positive sign and small in magnitude. Both Δ and Λ Ru stabilize DNA to essentially the same extent. Since by eq 1, the shift in T_m is seen to be a function of K and n, this finding emphasizes that there is little difference in the affinity of the isomers for DNA. We caution that the above analysis has neglected possible interaction of Δ and Λ Ru with single-stranded DNA, which could be appreciable by electrostatic attraction alone. The above estimates of K are thus *upper limits* and would be reduced in magnitude if Δ and Λ Ru bind to single-stranded DNA.

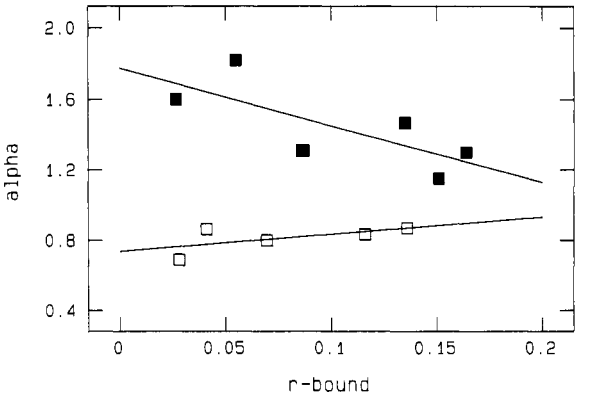

FIGURE 7: Results of competition dialysis experiments designed to assess the base specificity of Δ Ru (filled squares) and Λ Ru (open squares) in buffer D. The lines are linear least-squares fits to the data, yielding intercept value of 1.8 for Δ Ru and 0.7 for Λ Ru.

Figure 7 shows the results of competition dialysis experiments designed to quantitatively assess the base specificity of Δ and Λ Ru. The DNAs *C. perfringens* (31% GC) and *M. lysodeikticus* (72% GC) were used in these experiments. In the competition dialysis experiment, identical concentrations of each of these DNA samples are dialyzed against a common ligand solution. At equilibrium, the free ligand concentration is identical in all dialysis chambers. If the ligand binds preferentially to a DNA site, more ligand will partition into the chamber containing the DNA with a greater frequency of that site. Ligands that prefer GC base pairs will accumulate more in the *M. lysodeikticus* DNA sample, while ligands that prefer AT pairs will accumulate in the *C. perfringens* DNA sample. The results may be quantified using parameter $\alpha = r_{ml}/r_{cp}$, where r_{ml} and r_{cp} are the molar binding ratios of ligand to *M. lysodeikticus* and *C. perfringens* DNA, respectively. The limit of α as $r \rightarrow 0$ defines α_0, a quantity that provides a firm estimate of the base composition of the preferred ligand binding site (Mueller & Crothers, 1975; Chaires, 1992). From the data of Figure 7, Δ Ru is seen to accumulate in the GC-rich DNA sample, and $\alpha_0 = 1.8$ is found. For this DNA pair, that value is most consistent with the simplest type of base specificity, the requirement for a single GC base pair at the preferred binding site, for which $\alpha_0 = 2.3$ is predicted. In contrast, Λ Ru interacts preferentially with AT base pairs, with $\alpha_0 = 0.7$, a value most consistent with a base specificity that requires a single AT base pair at the preferred site. The two isomers clearly differ in their base specificity, although the specificity of each isomer is modest in comparison to other DNA binding ligands, such as actinomycin, daunomycin, and netropsin (Chaires, 1992).

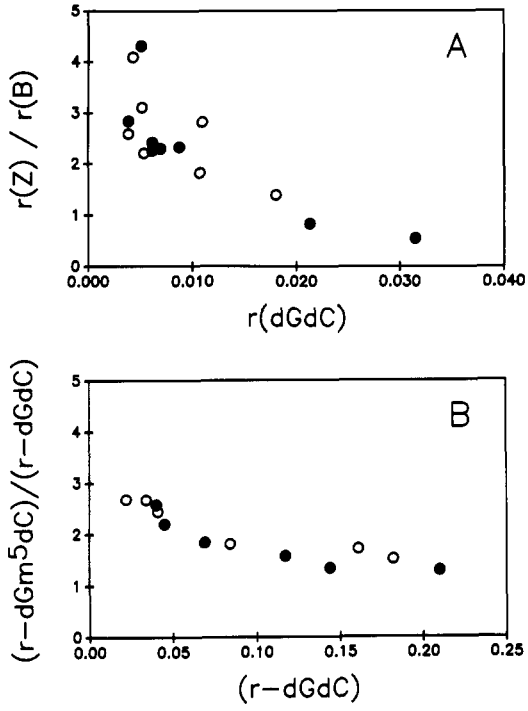

FIGURE 8: Results of competition dialysis experiments designed to assess the structural specificity (B versus Z) of Δ and Λ Ru in buffer D. (A) Ratio of bound compound for competitive binding to poly(dGm^{5}dC) and poly(dGdC) under solution conditions that induce the Z conformation in the methylated polymer, while the B conformation in the unmethylated polymer is maintained. (B) Competitive binding under conditions in which both polymers are in the B form. In both panels, open circles refer to Λ Ru and closed circles refer to Δ Ru.

Competition dialysis was also used to quantitatively assess the relative specificity of Δ and Λ Ru for B and Z DNA (Figure 8). In this experiment, competition was between poly(dGm^{5}dC) and poly(dGdC) in solutions containing 5 mM $MgCl_2$, conditions which favor the Z form of the former polymer and the B form of the latter. The polymer conformations in the experiment were confirmed using CD and UV absorbance spectroscopies. Figure 8A shows that more Ru *apparently* binds to the polymer in the Z conformation but that there is no difference between Δ or Λ Ru in this preference. As a control, a competition experiment between poly(dGm^{5}dC) and poly(dGdC) in solutions containing no $MgCl_2$ was conducted (Figure 8B). Surprisingly, the results of that experiment showed that both isomers bind preferentially to poly(dGm^{5}dC) (Figure 8B). The magnitude of the binding ratios as $r \rightarrow 0$ is essentially the same in both panels of Figure 8. The conclusion from these results is that neither Δ nor Λ Ru binds preferentially to Z form DNA but that both isomers preferentially bind to methylated DNA, regardless of its conformation. Collectively, these experiments indicate that the ruthenium isomers are not selective in any way for a rather extreme DNA conformational differences, i.e., B *versus* Z DNA.

The effect of *rac* Ru binding on the viscosity of rodlike DNA is shown in Figure 9. These results extend the binding range previously studied (Satyanarayana et al., 1992). Higher binding ratios in these experiments are attainable using the chloride salt of the racemate to prepare the concentrated titrant stock solution needed for the experiment. In previous studies (Satyanarayana et al., 1992), the solubility of the perchlorate salts of the purified enantiomers limited the range of binding ratios accessible in viscosity experiments. The effect of *rac* Ru on DNA viscosity is essentially the weighted average of

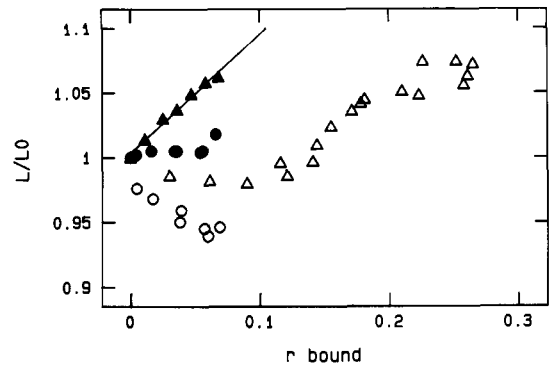

FIGURE 9: Effect of ethidium (closed triangles), *rac* Ru (open triangles), Λ Ru (closed circles), and Δ Ru (open circles) on the length of rod-like DNA.

the effect of the isolated isomers. Δ Ru decreases DNA viscosity, while Λ Ru exerts essentially no effect over the concentration range in which studies may be performed. The behavior of *rac* Ru in Figure 9 is both striking and unusual. At low binding ratios, DNA viscosity (length) is decreased, but upon further binding of the compound to DNA, viscosity (length) increases and reaches a plateau at a slightly higher level than observed for the DNA sample in the absence of added ligand. Such behavior is unusual but is not without precedent (Dattagupta et al., 1978). As will be discussed more fully in a later section, such behavior is consistent with bending or kinking of the helix upon binding *rac* Ru to DNA.

DISCUSSION

The principal conclusions we draw from the present and our previously published (Satyanarayana et al., 1992) studies are as follows: (i) Both Δ and Λ Ru bind to DNA by a single binding mode; (ii) Δ and Λ Ru bind, however, by different modes to DNA and consequently alter the hydrodynamic properties of DNA in different ways; (iii) Binding of both isomers to DNA is weak in comparison to that of other well-characterized DNA binding agents; (iv) Binding of both isomers to DNA is entropically driven and is predominantly electrostatic in nature; (v) Ru isomers each show a modest site specificity, with Δ Ru preferring GC bp and Λ Ru preferring AT bp; (vi) Neither isomer discriminates in any significant way between B and Z DNA.

Ruthenium Enantiomers Are Nonclassical DNA Binding Ligands. Our experience with Δ and Λ Ru leads us to conclude that they are unlike any of the well-characterized intercalating (ethidium, daunomycin, actinomycin) or groove binding (Hoechst 33258, netropsin, DAPI) agents but instead should be considered to be among a third class of "nonclassical" DNA binding agents. Among the latter class are the substituted diammonium cations studied by Gabbay and co-workers (Kapicak & Gabbay, 1975), the steroidal diamine irehdiamine A (Waring, 1970; Waring & Henley, 1975; Dattagupta et al., 1978), and the triphenylmethane dye crystal violet (Wakelin et al., 1981). These compounds all have structural features that make classical intercalation or groove binding either impossible or extremely unlikely. They typically have an overall nonplanar conformation that contrasts with the fused-ring aromatic structures of intercalating agents, or they have bulky substituents that would impart substantial steric hindrance and prevent both classical intercalation or groove binding (as exemplified by Hoechst, netropsin, and DAPI). Table II contrasts the physical properties of the Δ and Λ Ru DNA complexes with those of ethidium, daunomycin, and Hoechst 33258, compounds with proven DNA binding modes.

Distinctive differences may be seen for Δ and Λ Ru in their optical changes upon DNA binding and in their curious, and at first glance, contradictory effects on DNA unwinding and lengthening. It is in their effect on the hydrodynamic properties of DNA that Δ and Λ Ru reveal themselves, unambiguously, *not* to be classical intercalators, and which places them firmly among the nonclassical DNA binding agents.

Steric Hindrance in Δ Ru Binding to DNA. While Δ Ru unwinds DNA (Barton, 1983; Barton et al., 1984; Kelly et al., 1985), it *decreases* the viscosity of rodlike DNA (Satyanarayana et al., 1992; Figure 9). These properties are similar to those of the "bookmark" compounds studied by Gabbay and co-workers (Kapicak & Gabbay, 1975; Gabbay et al., 1972, 1973) and to those of crystal violet, a DNA binding dye (Wakelin et al., 1981). For steric reasons, neither of these compounds can fully intercalate into DNA, and binding is believed to occur by the *partial* insertion of a portion of the compound between adjacent DNA base pairs in a fashion that produces a kinked structure. For Δ Ru it is easy to see that the two nonintercalated phenanthroline rings, specifically the hydrogen atoms on positions 1 (8) of those rings, would block complete insertion of the third ring between the base pairs of DNA. Partial insertion for both isomers was described in molecular modeling and energy minimization calculations (Haworth et al., 1991), and the possibility that Δ Ru produced a kinked DNA structure was noted in the linear dichroism measurements of Hiort et al. (1990).

Δ Ru May Kink DNA. The notion that DNA kinking or bending results from Δ Ru binding is strongly reinforced by comparison of the hydrodynamic data presented in Figure 9 with similar data obtained for irehdiamine A (Dattagupta et al., 1978). Both compounds show the same qualitative behavior. At low binding ratios, a *decrease* in DNA length is observed, but as binding increases, a length *increase* is seen. Eventually, a plateau is reached that corresponds to a length slightly longer than observed for the DNA in the absence of added ligand. Dattagupta et al. (1978) proposed that such behavior could result from the introduction of kinks in the DNA helix. At low binding ratios, only a few kinks are introduced, changing the direction of the DNA helix axis, resulting in a decrease in its hydrodynamic length. As more kinks are introduced with increased binding, a rod-like superhelical structure would result since the kinks would not be phased, producing a structure that should behave hydrodynamically much like the starting DNA molecule. We believe this same explanation may be applied to the results presented in Figure 9 and interpret these data as evidence for DNA kinking. Although the results were obtained using *rac* Ru, our studies have shown that Λ Ru does not alter DNA length, so we attribute the effects seen for the racemate to the Δ isomer present in the mixture.

DNA Unwinding Is Not a Definitive Criterion for Intercalation. Previous studies showed that the ruthenium compounds unwind DNA, prompting the suggestion that they bind by intercalation (Barton, 1983; Barton et al., 1984; Kelly et al., 1985). DNA unwinding is not a definitive indication that intercalation has occurred (Dougherty & Pilbrow, 1984). The dicationic steroid irehdiamine A and its analogs offer the best proof of that assertion. These compounds have structures which do not allow intercalation, but they are able to unwind DNA (Waring & Henley, 1975; Waring, 1970) but do not increase the length of DNA in the manner predicted by the classical intercalation model (Waring, 1970; Dattagupta et al., 1978). In the absence of crystallographic structural data, we regard DNA lengthening as the most stringent requirement for proof of classical intercalation. Another supporting example, for which structural data exists, is the antitumor drug cisplatin. Cisplatin does not intercalate, but rather binds covalently in the DNA major groove by attachment to N(7) of guanine (Sherman et al., 1985). Nonetheless, cisplatin was reported to unwind closed circular DNA (Cohen et al., 1979; Scovell & Kroos, 1982; Blatter et al., 1984).

Optical Changes of Ru Isomers upon DNA Binding Are Unlike Those Observed for Proven Intercalators. Inspection of Table II reveals that the changes in the optical properties of Δ and Λ Ru upon DNA binding are of small magnitude and are unlike changes observed for both proven intercalators and groove binding agents. Such slight optical changes were noted also by Hiort et al. (1990), who argued that such changes could not arise from true intercalators, a point with which we agree. The observed optical changes reflect the fact that, structurally, the ruthenium isomers are unusual DNA binding ligands. The compounds possess three phenanthroline rings only one of which could potentially intercalate between the base pairs of DNA. This feature makes it difficult to apply certain criteria normally used to determine a ligand's binding mode. For example, a shift in the absorption spectrum of a compound in the presence of DNA is often used as evidence that intercalation has occurred (Dougherty & Pilbrow, 1984). It is well-known that the bands in the visible region of the spectrum, of Δ and Λ Ru, are due to metal-to-ligand charge transfer MLCT transitions (Balzani et al., 1975) involving all three phenanthroline rings. If one ring were to intercalate, the symmetry of the compound would be reduced from D_3 in the free ion to pseudo-C_2 for the intercalation complex. If the electronic perturbation by DNA on the intercalated ring were sufficiently strong, the reduction in symmetry would be evident by the presence of additional bands in the absorption spectrum. As is evident from earlier published spectra (Barton et al., 1984; Hiort et al., 1990) and those presented in Figure S1 (supplementary material), this is clearly not the case for either isomer in the presence of DNA. Elfing and Crosby (1981) explored the effects of reduction in molecular symmetry on the photoluminescence and electronic structure of tris bidentate ruthenium(II) compounds. They concluded that unless the perturbation toward C_2 symmetry is substantial, as would occur with cis[$Ru(CN)_2(phen)_2$], where one phenanthroline is replaced by two strong field cyanide ligands, various substituted ruthenium(II) complexes having C_2 molecular symmetry photochemically exhibit higher trigonal, D_3, symmetry. Thus, unlike the case of single chromophore intercalating ligands (such as ethidium bromide and daunomycin), assigning the binding mode from alterations in the absorption spectra for a compound which has three symmetry equivalent chromophores, only one of which can undergo a significant perturbation in the presence of DNA, is a much more difficult task.

Fluorescence Emission Can Be Used To Establish the Number of Bound Species but Not the Mode of DNA Binding. Both Δ and Λ Ru show slight enhancements in steady-state fluorescence emission upon DNA binding (Table I; Figure S3). The magnitude of these effects are again much less than observed for compounds of proven binding modes (Table II). Compounds with distinctly different binding mechanisms, e.g., groove binders and intercalators, can exhibit fluorescence changes in the presence of DNA which are comparable in magnitude and sign (Hard et al., 1990; Olmsted & Kearns, 1977; Barcellona & Gratton, 1990). While fluorescence emission may certainly be used to monitor binding events, it

is therefore of no use whatsoever in attempting to infer the *mode* of ligand binding to DNA. Singular value decomposition may, however, be used to analyze fluorescence emission data to explore the possible multiplicity of DNA binding modes. SVD is a rigorous and model-free analytical tool for this task. The results of SVD analysis of fluorescence emission spectra we obtain (Figure S4, supplementary material) provide firm evidence for a *single* DNA binding mode for both Δ and Λ Ru and evidence that fluorescence emission of these compounds in the presence of DNA is therefore a linear combination of emission from the free and *one* bound form. Binding of Ru isomers to DNA is therefore a simple two-state process.

Fluorescence Quenching Studies Show That Δ and Λ Ru Bind to DNA, but Cannot Determine the Mode of Binding. The fluorescence quenching experiments shown in Figure 3 indicate that when bound to DNA, both Δ and Λ Ru are inaccessible to the anionic quencher $K_4[Fe(CN)_6]$. When the amount of DNA is in large excess relative to ligand (e.g., 500:1), the Stern–Volmer quenching constant is reduced by at least a factor of 40. At lower DNA to isomer ratios, biphasic Stern–Volmer plots are observed (Figures 3 and 4), arising from the quenching of a mixture of free and a *single* bound species. Nonlinear least-squares analysis may be used to quantitatively evaluate the contributions of these two species. Because of the fact that both Δ and Λ Ru carry a net charge of 2+, quenching experiments must be conducted at constant ionic strength. If this precaution is not taken, addition of $K_4[Fe(CN)_6]$ perturbs the Ru–DNA equilibrium, and artifactual Stern–Volmer plots result, as may be clearly seen in Figure 4. Zinger and Geacintov (1988) have shown that bulky external quenching agents cannot be used to differentiate DNA binding modes, presumably because access into DNA grooves is sterically hindered. Only O_2 was found to be useful in distinguishing between a known intercalator and a known groove binding agent (Zinger & Geacintov, 1988). Since $[Fe(CN)_6]^{4-}$ is a bulky, negatively charged quencher, it would certainly experience both steric hindrance and electrostatic repulsion upon attempting to penetrate DNA grooves. We conclude, therefore, that the data of Figures 3 and 4 show definitively that Δ and Λ Ru bind to DNA but reveal nothing about the *mode* (i.e., intercalation vs groove binding) of binding. This conclusion differs substantially from previous interpretations of fluorescence quenching studies of this same system (Barton et al., 1982, 1984, 1986).

Binding of Δ and Λ Ru to DNA Is Entropically Driven. The thermal difference spectra in Figure 5 indicate that the enthalpy for the interaction of both Δ and Λ Ru with DNA is small in magnitude and positive in sign. DNA binding for both isomers is therefore *entropically* driven. Since neither ruthenium isomer possesses hydrogen bond donor or acceptor sites, it would appear that van der Waals contacts, electrostatic interactions, and/or effects related to the solvation of DNA are responsible for the observed enthalpy changes. The similarities in enthalpy, free energy (binding constant), and entropy for Δ and Λ Ru are difficult to understand in terms of their apparently different binding modes, as inferred from their differing effects on DNA hydrodynamics. We have previously shown (Satyanarayana et al., 1992), however, that binding of both Δ and Λ Ru to DNA is predominantly electrostatic in nature. The new finding that binding of both isomers is entropically driven is consistent with an electrostatic binding mode.

Base Specificity of Δ and Λ Ru Binding to DNA. Competition dialysis offers a simple and direct way of quantitatively evaluating the base specificity of ligand binding to DNA (Mueller & Crothers, 1975; Chaires, 1992). By that method, we find that Δ and Λ Ru each exhibit a modest base specificity. GC base pairs are the preferred site of Δ Ru binding, while AT base pairs are the preferred site of Λ Ru binding. In both cases, the binding preference is not absolute, and the magnitude of the effect is weak in comparison to other DNA binding agents (Chaires, 1992). The base specificities we infer for Δ and Λ Ru by the competition dialysis method are in agreement with previous binding studies utilizing natural DNA samples of varying GC content and synthetic polydeoxynucleotides (Barton et al., 1986; Hiort et al., 1990).

Ruthenium Isomers Do Not Discriminate B DNA from Z DNA. Part of the initial excitement over the enantiomers of $[Ru(o\text{-phen})_3]^{2+}$ was that they could potentially discriminate against, and therefore probe, differing DNA secondary structures (Barton, 1986). The competition dialysis results presented in Figure 8 show, in the most direct and simple way possible, that there is no stereoselective binding of either Δ or Λ Ru to Z DNA. Neither Δ nor Λ Ru is therefore of practical use as a probe for Z DNA. Hard et al. (1987) first arrived at the same conclusion by an independent experimental approach, a conclusion that is reinforced by the results presented here. A recent publication claims that $[Ru(o\text{-phen})_3]^{2+}$ enantiomers (and compounds derived from these) intercalate into Z DNA (Friedman et al., 1991). We believe that claim to be wholly without merit, for two reasons. First, only photophysical criteria were used to establish the mode of binding, criteria that cannot, by themselves, definitively establish the mode of binding of DNA ligands. Second, a number of ligands have been shown to allosterically convert Z DNA to an intercalated right-handed form (Chaires, 1985, 1986a,b; Walker et al., 1985a,b; Lamos et al., 1986). At low binding ratios, ligand binding can convert local regions from the Z to the right-handed form, and thus ligand may be bound to right-handed DNA while the rest of the polymer remains in a left-handed conformation. Friedman et al. (1991) neglected to consider this possibility and did not provide control experiments to eliminate it as a simple explanation of their observations. Since we have shown that neither Δ nor Λ Ru binds to B DNA by classical intercalation, we doubt the claim that they intercalate into Z DNA. Finally, a comparison of the relative affinities of proven intercalators for B and Z DNA is of interest to contrast the insignificant selectivity exhibited by Δ and Λ Ru. Daunomycin and adriamycin bind 30 and 90 times as tightly, respectively, to B DNA than to Z DNA (Chaires, 1986a,b). The simple intercalator ethidium binds 300 times more tightly to B DNA than to Z DNA (Walker et al., 1985a). Actinomycin D binds 1000 times more tightly to B DNA than to Z DNA (Walker et al., 1985b). These quantitative studies show unambiguously that B form DNA is the preferred conformation for the binding of proven intercalating compounds. Quantitative studies of the allosteric conversion of Z DNA to the right-handed form by groove binders have not been reported, although netropsin has been shown to convert poly(dGdC) from the Z to the B form (Zimmer et al., 1983).

Ru Enantiomers as Probes of DNA Structure. The potential of Ru enantiomers as specific probes of DNA conformation appears limited. The compounds bind weakly to DNA, have only a modest base specificity, and cannot discriminate against radically different conformations of DNA (i.e., B vs Z DNA). In order for an agent to be a useful probe, it must have a high affinity for a *specific* type of site in the presence of a large number of competing nonspecific sites. The thermodynamic requirements that define "specific"

binding in such a situation have been discussed by von Hippel and Berg (1987), and it is clear that binding sites with the requisite high affinity to qualify as "specific" have yet to be discovered or characterized for either Δ or Λ Ru. The fact that Δ and Λ Ru bind weakly and with little specificity to DNA may, however, make them useful and attractive as nonspecific photofootprinting reagents. Irradiation into the MCLT band in the visible region of the spectrum of ruthenium compounds is known to produce DNA strand breakage via a mechanism involving singlet oxygen (Kelly et al., 1985; Fleisher et al., 1986; Tossi & Kelly, 1989). This useful photochemistry, coupled with their weak, relatively nonspecific DNA binding, could make Ru enantiomers useful adjuncts to DNase I as tools for measuring the binding of sequence specific ligands to DNA in footprinting experiments.

CONCLUSIONS

Our studies show that Δ and Λ Ru bind only weakly to DNA by a predominantly electrostatic, entropically driven binding mode. Each enantiomer binds to DNA by a single binding mode, and neither binds by classical intercalation. The selectivity of enantiomer binding to DNA is at best weak. There is a modest stereoselectivity of their DNA binding, with the Δ isomer binding somewhat better toward B DNA than the Λ isomer. There is a base specificity of isomer binding, with Δ Ru preferring GC base pairs and Λ Ru preferring AT base pairs. Neither isomer discriminates Z DNA from B DNA. Our studies lead us to conclude that, in spite of their initial promise, neither Δ nor Λ Ru appears particularly useful as a practical probe of DNA sequence or conformation.

SUPPLEMENTARY MATERIAL AVAILABLE

Figures S1–S3 showing the primary data used to compile Table I and Figure S4 showing the results of SVD analysis of fluorescence emission spectra (4 pages). Ordering information is given on any current masthead page.

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