Interaction of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ with DNA: A Calorimetric and Equilibrium Binding Study

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Abstract: Fluorescence and absorption spectroscopy, isothermal titration calorimetry, and viscosity measurements have been used to characterize the interaction of Δ and Λ [Ru(phen)$_2$DPPZ]$^{2+}$ with calf thymus DNA. The method of continuous variations revealed two distinct binding stoichiometries for both Δ- and Λ-DPPZ, corresponding to 0.7 and 3 mol of base pair/mol of ligand. Binding isotherms were obtained for the two enantiomers, both of which show strong binding to DNA, with $K = 3.2 \times 10^6$ M^{-1} bp and 1.7×10^6 M^{-1} bp for the Δ and Λ isomers, respectively, at 25 °C in solutions containing 50 mM NaCl. Titration calorimetry gave ΔH values of +0.3 kcal mol^{-1} for Δ-DPPZ and +2.9 kcal mol^{-1} for Λ-DPPZ for their interaction with DNA. These small positive enthalpies, which were confirmed using thermal difference spectroscopy, indicated that the binding of these compounds to DNA is entropically driven. An enthalpy of +2.5 kcal mol^{-1} was obtained for the binding of the parent compound, tris(phenanthroline)-Ru(II), to DNA. Titration of all three compounds into buffer gave a nonnegligible heat of dilution. The salt dependence of the binding constant was examined for both isomers. The slope $\mathbf{SK} = (\delta \log K/\delta \log[Na^+])$ was found to be 1.9 and 2.1 for the Δ and Λ isomers, respectively. By using polyelectrolyte theory to interpret the observed salt dependence of the equilibrium constant, it can be shown that there is a significant nonelectrostatic contribution to the binding constant. Relative viscosity experiments showed that both Δ- and Λ-DPPZ increase the length of rod-like DNA, in a manner consistent with binding by classical intercalation. Fluorescence energy transfer experiments provided additional evidence for the intercalation of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ into DNA.

Introduction

Over the past decade there has been substantial interest in the DNA binding properties of a number of ruthenium(II) complexes,[1-12] in the hope of developing novel probes of DNA structure or new therapeutic agents. Ruthenium complexes containing planar aromatic ligands that bind to DNA have many convenient features, including the ease with which the ligand can be attached to the metal in a controlled manner, strong visible absorbance, due to a localized metal-to-ligand charge transfer[4] (MLCT), and strong fluorescence emission. These latter properties provide a convenient handle for monitoring the DNA binding process.

The parent compound for many of these studies is tris-(phenanthroline)Ru(II). The binding of this metal complex to DNA has been actively studied,[2,6,7] although its exact DNA binding mode remains an area of intense controversy. Barton *et al.* have proposed that binding occurs through two mechanisms: (1) intercalation, based on the observation that binding of Ru(phen)$_3$ (tris(1,10-phenanthroline)ruthenium(II)) causes the DNA duplex to unwind, and (2) "surface" interactions.[2,6-8] They have obtained a binding constant of 6.2×10^3 M^{-1} for the racemate binding to calf thymus DNA. Satyanarayana *et al.* have used viscosity measurements to examine the effects of binding on the hydrodynamic properties of the duplex. They argued that since neither enantiomer of Ru(phen)$_3$ lengthened short, rod-like DNA, classical intercalation could not be the binding mode.[9] Furthermore, molecular modeling and energy minimization calculations suggested that there is, at best, only partial insertion of the phenanthroline ring betwen base pairs.[10] NMR and CD data show that interaction of the ligand with DNA occurs primarily in the minor groove, and that binding is *not* by classical intercalation, but rather by the insertion of two phenanthroline rings into the minor groove, leading to slight distortions of DNA structure.[11]

Many new structural analogs based on Ru(phen)$_3$ have been

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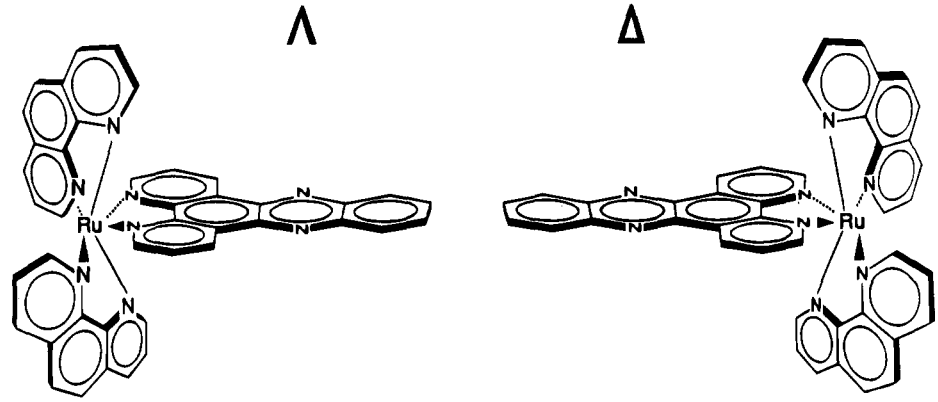

Figure 1. The molecular structures of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$.

synthesized, including Ru(bpy)$_3^{2+}$, [Ru(bpy)$_2$(DPPZ)]$^{2+}$ (DPPZ = dipyridophenazine), and [Ru(phen)$_2$DPPZ]$^{2+}$ (Figure 1). One of the first dipyridophenazine-containing complexes to be investigated was [Ru(bpy)$_2$DPPZ]$^{2+}$. This compound was proposed to act as a molecular "light switch" for DNA because it lacks luminescence in aqueous solutions, but it shows intense luminescence in the presence of DNA. Based on an observed DNA unwinding angle of 30 ± 11°, an intercalative binding mode was suggested.[1] Anionic quenching experiments were used to demonstrate that upon binding to B form DNA both [Ru(bpy)$_2$DPPZ]$^{2+}$ and [Ru(phen)$_2$DPPZ]$^{2+}$ exhibit a biexponential decay in emission, and this was interpreted to result from two separate binding modes.[3] The variation of emission characteristics with DNA conformation has also been investigated. The order of luminescent yield was found to be the following: triplex DNA > Z form > B form > A form. This order was proposed to reflect the level of protection afforded to the ruthenium−DPPZ excited state from quenching by water.

Hiort *et al.* synthesized enantiomerically pure Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ in order to study their interaction with calf thymus DNA.[12] Equilibrium binding constants for both isomers were found to be around 10^8 M^{-1} in solutions containing 10 mM NaCl. The complex of bound Δ was found to give a relative quantum yield 6 to 10 times greater than the Λ-DNA complex. A negative linear dichroism of the DPPZ ligand transition at 380 nm supported intercalation as the binding mode for both isomers. These workers found that there were two distinct luminescent lifetimes for each enantiomer when bound to DNA. The longer lifetime fraction was found to increase with binding ratio, hence the existence of the two lifetimes was explained not as two separate binding modes but as being due to the distribution of intercalated complexes along the DNA helix, so that ligands bound contiguously have longer lifetimes. Eriksson and co-workers have studied the interaction of Δ-[Ru(phen)$_2$DPPZ]$^{2+}$ with the oligonucleotide [CGCGATCGCG]$_2$ using NMR.[11] These data indicate that the binding kinetics are in the intermediate exchange range and are thus slower than the parent compound [Ru(phen)$_3$]$^{2+}$. This observation is consistent with stronger binding of the DPPZ ligand to DNA (relative to Ru(phen)$_3$) and with intercalation.

Studies of the interaction of Ru compounds with DNA have, to date, been concerned largely with establishing their mode of binding and with the possible structure of their DNA complexes. Apart from the determination of binding constants, the thermodynamics of their DNA binding has not been studied in any detail. The aim of this present study is to determine the complete thermodynamic profile (ΔG, ΔH, and ΔS) for the interaction of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ with DNA. In this study we have determined the binding constant for the interaction of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ with calf thymus DNA using fluorescence titration techniques and have examined the salt dependence of the binding constant. These studies allow us to dissect the binding free energy into its electrostatic and nonelectrostatic contributions by the application of polyelectrolyte theory. The method of continuous variations was used to show that both isomers bind to DNA with two distinct stoichiometries. One corresponds to 3 mol of base pairs per mol of ligand, and the other is equivalent to 0.7 mol of base pair per mol of ligand. The former value is typical of an intercalator. We also present data for the direct measurement of ΔH using isothermal titration calorimetry. This has allowed us to elucidate the enthalpic and entropic contributions to $\Delta G°$. The binding modes of Δ- and Λ-DPPZ to DNA have been examined using viscosity measurements, since increases in the length of duplex (and hence increases in viscosity) are a key diagnostic of classical intercalation. The DNA binding mode was further probed by fluorescence energy transfer experiments, which provided strong additional support for an intercalative binding mode for both Δ- and Λ-DPPZ. On the basis of the data presented in this study it is possible to discuss the mode, strength, and detailed energetics of the interaction between [Ru(phen)$_2$DPPZ]$^{2+}$ and DNA.

Experimental Section

Materials. The synthesis, separation, and purification of the [Ru(phen)$_2$DPPZ]$^{2+}$ isomers as their chloride salts were carried out as previously described by Hiort et al.[12] Concentrations of the metal complexes were determined by measuring the visible absorption at 439 nm and by using a molar extinction coefficient, ϵ_{439} = 20 000 M^{-1} cm^{-1}. Calf thymus DNA was purchased from Pharmacia (Lot No. 27-4562-02) and was sonicated and purified as described earlier.[13] Before further use the DNA was dialyzed in the appropriate buffer for 24 h. Experiments were carried out in a buffer consisting of 5 mM Tris−HCl, 50 mM NaCl, pH 7.1, unless noted otherwise.

Instrumentation. Fluorescence measurements were made using a Perkin-Elmer 650-40 fluorescence spectrophotometer with a xenon lamp and a 515 nm cutoff filter or using a I.S.S., Inc., Greg 200 photon counting fluorimeter. Absorbance spectra were recorded using a Varian Cary 3E UV−visible spectrophotometer linked to a Peltier heating temperature control accessory and interfaced to a Gateway 386 PC for data collection and analysis. Calorimetric data were obtained using a Hart Scientific isothermal microtitration calorimeter linked to a Gateway 2000 PC.

Continuous Variation Analysis. Binding stoichiometries were obtained for the two DPPZ enantiomers using the method of continuous variation.[14] The concentration of both metal complex and DNA was varied, while the sum of the concentrations of the two reactants was kept constant at 80 μM (in terms of base pairs for the DNA). Varying

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volumes of equally concentrated stock solutions of both reactants were mixed together to give a final volume of 250 μL and mole fractions of DPPZ ranging from 0.05 to 1. The fluorescence intensities of these mixtures were measured at 25 °C using an excitation wavelength of 439 nm and collecting all emitted light passing through a 515 nm cutoff filter. The experiment was repeated using the same stock solution of ligand but replacing the DNA with Tris−HCl, 50 mM NaCl buffer. The difference in fluorescence (ΔF) was plotted against the mole fraction of drug.

Determination of Binding Constants. Binding isotherms were obtained by carrying out fluorescence titrations in which three different but fixed concentrations of Δ- and Λ-DPPZ were titrated with calf thymus DNA ranging in concentration from 10^{-9} to 10^{-3} M bp. The concentration of the DPPZ isomers was fixed at 1, 5, and 10 μM. The excitation wavelength was set at 439 nm and all emitted light passing through a 515 nm cutoff filter was collected. The mixture of DNA and metal complex in the cuvette was continuously mixed using a motorized stirrer in order to ensure a homogeneous distribution of components and to prevent photobleaching. All titrations were carried out at 25 °C. Fluorescence titration data were fit directly to obtain binding constants, using a fitting function incorporated into FitAll (MTR Software, Toronto, Canada). Briefly, the observed fluorescence is assumed to be a sum of the weighted contributions of free and bound ligand:

$$F = F_0(C_t - C_b) + F_bC_b \quad (1)$$

where F is the apparent fluorescence at each DNA concentration, F_0 is the fluorescence of free ligand, and F_b is the fluorescence of the bound species. For the interaction of a ligand D with a DNA site S, it may be easily shown that:

$$Kx^2 - x(KS_0 + KD_0 + 1) + KS_0D_0 = 0 \quad (2)$$

where $x = C_b$, K is the association constant, S_0 is the total site concentration, and D_0 is the total ligand concentration. Equation 2 is readily solved using the quadratic formula. Data in the form of fluorescence response F as a function of total DNA site concentration at fixed concentration of ligand may then be fit by nonlinear least-squares methods to obtain K, F_0, and F_b.

Determination of the Salt Concentration Dependence of the Equilibrium Binding Constant. A sample of DNA−metal complex in which 70% of the ligand was initially bound was prepared in Tris−HCl, 50 mM NaCl buffer, and was used to perform a reverse salt titration.[15] This was carried, out by placing 1.5 mL of the sample into a cuvette and recording the fluorescence emission. A 5 M NaCl solution was then titrated into the sample in 2 μL aliquots and the decrease in fluorescence emission was monitored up to a maximum salt concentration of 0.2 M. A decrease in fluorescence intensity as a result of increasing Na^+ concentration occurs because the salt causes the dissociation of the DPPZ from the duplex changing the distribution of free and bound drug. Knowing the relative fluorescence values of the free and bound forms of the drug, the concentrations of free (C_f) and bound (C_b) ligand may be determined at each step in the titration. It is then possible to determine the binding constant (K) at each salt concentration using the neighbor exclusion model of McGhee and von Hippel.[16] The data were plotted as log$K$ against log[Na^+]. The slope of this graph gives an estimate of $\mathbf{SK} = (\delta \log K/\delta \log[Na^+])$. This parameter can then be used to dissect the binding free energy into its electrostatic and nonelectrostatic components using polyelectrolyte theory.[17]

Thermal Difference Spectra. These measurements were carried out using a 50% saturated sample of DNA/DPPZ complex. Absorption spectra were recorded, using buffer in the reference compartment, between 300 and 600 nm, at 10, 25, and 45 °C. The spectra recorded at 25 °C were subtracted from the spectra recorded at the higher and lower temperatures to give two difference spectra.

Isothermal Titration Calorimetry. Grenthe *et al.* have reported the enthalpy changes for the ionization of Tris over a wide range of temperature.[18] These data were used to calibrate the calorimeter before making any measurements with DNA. The protonation of a 0.02 M solution of Tris at 25 °C using a standard 0.0997 N hydrochloric acid solution gave a change in enthalpy of −11.35 kcal mol^{-1}; this value is in excellent agreement with the published figure of −11.34 kcal mol^{-1}. Stock solutions of both titrate (DNA) and titrant (Δ- or Λ-DPPZ) were prepared so that the DNA would always be in excess, thus ensuring that all the ligand titrated into the calorimeter would bind. Typically DNA concentrations of 2.3 mM bp were used with drug concentrations of 0.7 mM. Having allowed the DNA to equilibrate at 25 °C in the sample cell, the titrant was added in 5 μL aliquots 500 s apart until a total of 75 μL had been added. Data were collected as heat (μJ/s) against time (s). Each addition of ligand produced a peak that could be integrated to give a value for ΔH. Both isomers of DPPZ were found to have a significant heat of dilution. The magnitude of this heat was determined by titrating the ligands into Tris−HCl, 50 mM NaCl buffer. The heat of dilution was subtracted from the value of ΔH determined by titration into DNA to give a corrected value for the change in enthalpy. All titration series were repeated at least three times, and averages were calculated from the combined results. Each reported enthalpy value thus represents an average of approximately 40 individual determinations.

Viscosity Measurements. Viscosity experiments were carried out using a Cannon−Manning semi-micro viscometer maintained at a constant temperature of 25 °C in a VWR circulating water bath, using protocols described previously.[9] The viscometer required 300 μL of sample and the flow time for the BPE buffer (8 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) used in these experiments was 374.53 (±0.34) s. In order to minimize contributions to the viscosity due to changes in DNA persistence length, sonicated calf thymus DNA was used whose average length was 200 bp. DNA of this length behaves like a stiff rod. The concentration of DNA was 9.5×10^{-5} M bp, and samples were prepared by adding ligand to the DNA so as to give total ligand/bp ratios of 0.07, 0.15, and 0.3. The flow times of samples were measured after a thermal equilibration time of 30 min. Each sample was measured four times and an average flow time was calculated. Data were presented as $(\eta/\eta^0)^{1/3}$ *versus* binding ratio,[19] where η is the viscosity of DNA in the presence of ligand and η^0 is the viscosity of DNA alone. Viscosity values were calculated from the observed flow times of DNA-containing solutions (t) corrected for the flow time of buffer alone (t_0), $\eta = t - t_0$.

Fluorescence Contact Energy Transfer. Contact energy transfer from DNA bases to bound ligand[20−22] was measured from corrected excitation spectra recorded from 240 to 350 nm at 1 nm intervals. The ratio between the quantum yield of bound ligand with excitation in the UV spectral region (Q_λ) to that at 310 nm (Q_{310}) was calculated from the expression:

$$\frac{Q_\lambda}{Q_{310}} = \left[\frac{I_\lambda E_{310}}{I_{310}E_\lambda}\right]_b \left[\frac{I_{310}E_\lambda}{I_\lambda E_{310}}\right]_f$$

where I and E are respectively the measured fluorescence and molar extinction coefficient at wavelengths λ and 310 nm, and the subscripts b and f refer to the bound and free forms of the ligand.[20−22] The wavelength 310 nm was chosen for the normalization because of the negligible absorbance of DNA in that region of the spectrum. Excitation spectra were corrected for the inner filter effect prior to normalization.

Results and Analysis

Continuous Variation Analysis. Figure 2 shows Job plots for Δ- and Λ-$[Ru(phen)_2DPPZ]^{2+}$. The first point of intersection corresponds to a drug mole fraction of 0.25 for Δ and 0.26

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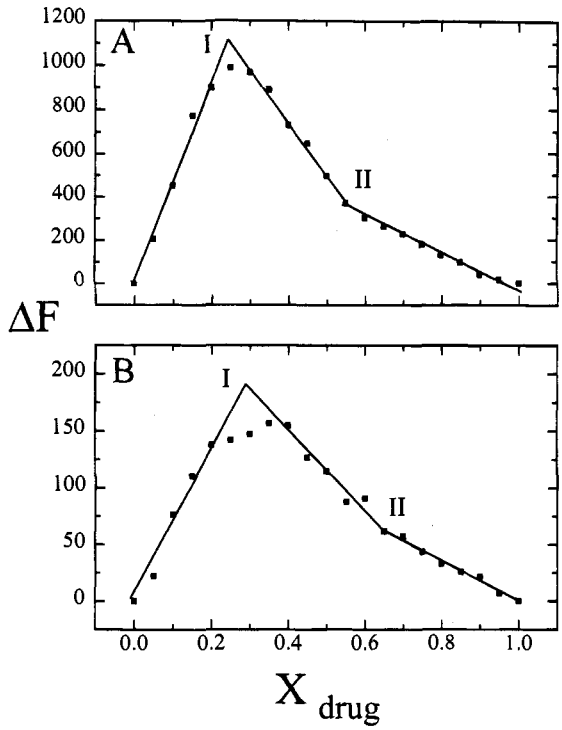

Figure 2. Job plots for the binding of (A) Δ- and (B) Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ to calf thymus DNA at 25 °C and in Tris−HCl, 50 mM NaCl, pH 7.1. The concentration of drug plus DNA was constant at 80 μM. Fluorescence measurements were made with an excitation wavelength of 439 nm; all emitted light passing through a 515 nm cutoff filter was collected. The *y* axis, Δ*F*, represents the difference in fluorescence between mole fractions of drug in DNA and drug in buffer. Crossover points were determined by adding lines of best fit to each portion of the data using linear least-squares analysis. The two points of inflection are X_{drug} = 0.25 (I) and 0.58 (II) for Δ-DPPZ and X_{drug} = 0.26 (I) and 0.68 (II) Λ-DPPZ.

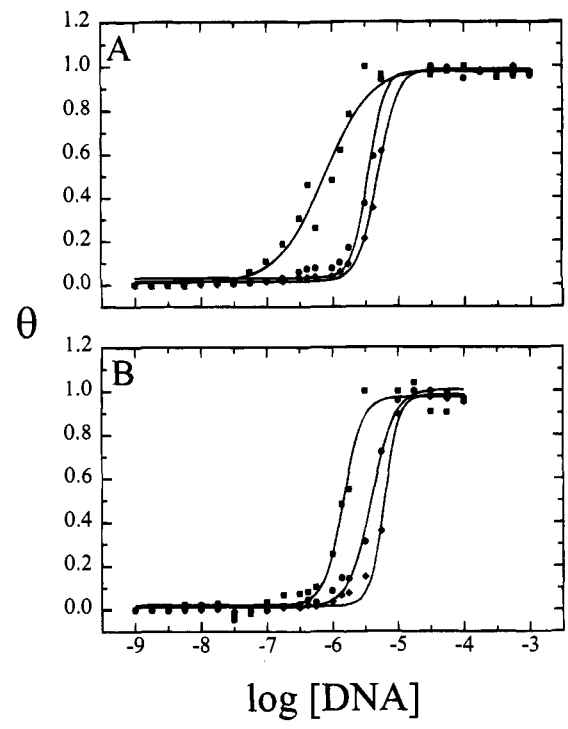

Figure 3. Fluorescence titration for the interaction of (A) Δ- and (B) Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ with calf thymus DNA. The concentration of ligand was kept constant at (from left to right) 1, 5, and 10 μM, while the DNA concentration was varied between 1 mM and 1 nM bp. The quantity θ represents the fractional saturation and was calculated from the expression $\theta = (F - F_0)/(F_b - F_0)$, where *F* is the apparent fluorescence, F_0 is the fluorescence in the absence of DNA, and F_b is the fluorescence of fully bound ligand. Curve fitting and determination of binding constants were carried out by using nonlinear least-squares analysis as described in the text.

for Λ. This is equivalent to a stoichiometry of 3 mol of base pairs per mol of ligand and defines the primary site size of the Ru compound. The second point of intersection occurs at a drug mole fraction of 0.58 for Δ-DPPZ and 0.68 for Λ-DPPZ. This indicates that at the higher drug mole ratios, there is a second mode of binding corresponding to 1.4 and 2.1 mol (respectively) of ligand bound per mol of base pair. The exact nature of the binding mode that produces this unusual stoichiometry cannot be inferred from these studies alone, but it could result from stacking of ligand molecules on the DNA surface.

Equilibrium Binding Studies. Binding isotherms obtained for the interaction of Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ with calf thymus DNA by fluorescence titration of three different but fixed amounts of ligand with varying DNA concentrations are shown in Figure 3. For both enantiomers there was found to be a shift of the isotherm midpoint toward higher DNA concentration and a narrowing of the width of the isotherm with increasing ligand concentration. Such behavior occurs when the concentration of ligand is larger than the reciprocal of the binding constant. Examination of Figure 3 shows that there is a difference in position of the titration midpoints between the two enantiomers, with the midpoint occurring at a lower DNA concentration for the Δ isomer. It can be concluded from this observation that the Δ isomer binds more tightly to DNA than does the Λ isomer. Quantitative analysis of the binding data shows that for Δ-DPPZ, $K = 3.2\ (\pm 0.5) \times 10^6\ M^{-1}$ bp, and for Λ-DPPZ, $K = 1.7\ (\pm 0.2) \times 10^6\ M^{-1}$ bp. The Δ isomer binds 1.9 times more strongly than the Λ isomer, a difference in affinity which represents at best only a modest enantiomeric selectivity. We note that the conditions used in these titration experiments (excess DNA) would evaluate the binding constant for the process with a stoichiometry of 3 base pairs per drug.

The Salt Dependence of the Binding Constant. Figure 4 shows the dependence of *K* on the concentration of Na^+ as determined by reverse salt titrations. It is clear from this diagram that the binding constant decreases with increasing salt concentration. This is due to a stoichiometric amount of counterion release that accompanies the binding of a charged ligand.[17] It was found that the dependence of *K* on salt concentration becomes nonlinear at the higher ionic strengths. Slopes were obtained by linear fits of the data below 0.1 M NaCl. Such a procedure is justified since the polyelectrolyte theories used for subsequent analysis are based on limiting laws that are strictly applicable to salt concentrations lower than 0.1 M. It is possible to analyze these data using the polyelectrolyte theory of Record et al.[17] From that theory the slopes of the lines in Figure 4 are equal to the following:

$$\mathbf{SK} = \delta \log K / \delta \log[Na^+] = -Z\psi$$

where *Z* is the charge on the DPPZ molecule and Ψ is the fraction of counterions associated with each DNA phosphate (Ψ = 0.88 for double-stranded B form DNA). The quantity **SK** is equivalent to the number of counterions released upon binding of a ligand with net charge *Z*. For Δ- and Λ-DPPZ we find that respectively 1.9 and 2.1 counterions are liberated from the polymer upon binding of each ligand molecule. From these values of ZΨ the charge on each ligand is as follows: +2.1 for Δ-DPPZ and +2.3 for Λ-DPPZ. These values correspond quite well to the two positive charges carried by the [Ru(phen)$_2$DPPZ]$^{2+}$ ligands at neutral pH.

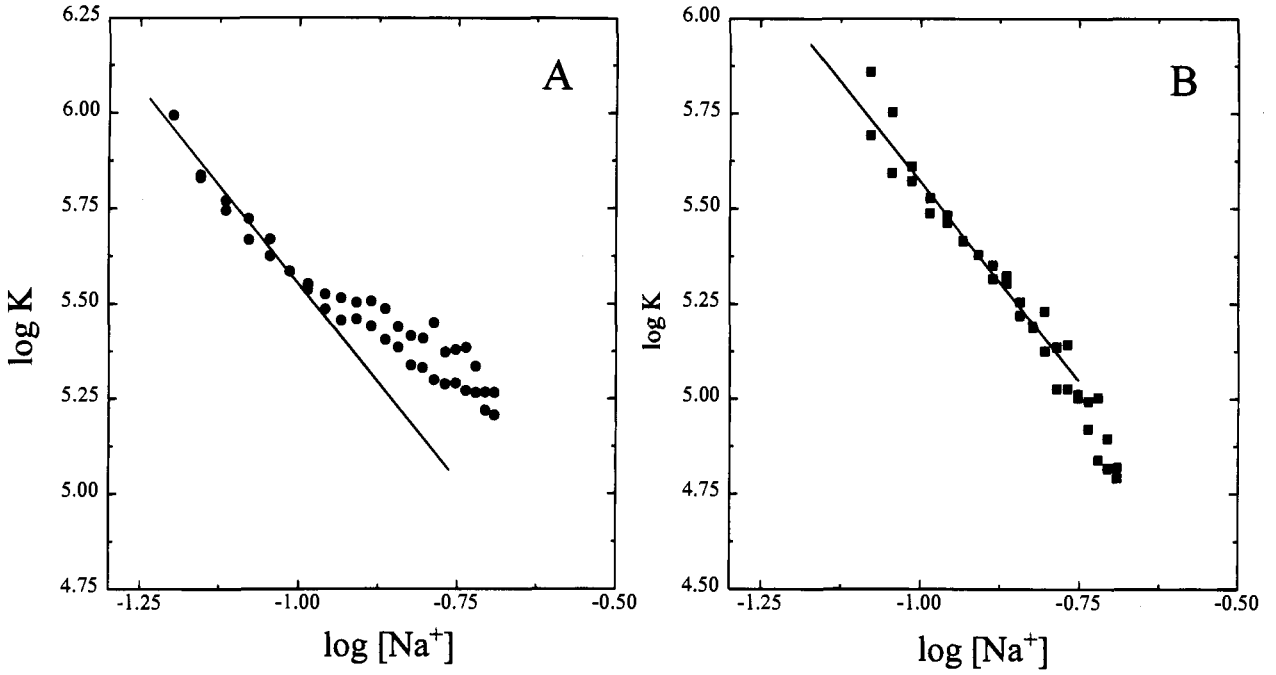

Figure 4. The variation of the binding constant (K_{obs}) for the interaction of (A) Δ- and (B) Λ-$[Ru(phen)_2DPPZ]^{2+}$ with calf thymus DNA as a function of salt concentration. The two sets of data on each graph refer to successive repeats of the reverse salt titration under identical conditions. Values for K_{obs} were obtained with a $[Na^+]$ range of 0.06 to 0.2 M. At the higher salt concentrations the data were found to deviate from linearity, hence in order to evaluate **S** the initial portion of the data was used to obtain the best linear least-squares fit. The values for the slopes are 1.9 for Δ-DPPZ and 2.1 for Λ-DPPZ.

Isothermal Titration Calorimetry. A direct determination for the binding enthalpy of Δ- and Λ-DPPZ to calf thymus DNA was achieved using isothermal titration calorimetry. Sample primary data from a titration are presented in Figure 5. The solid line is the titration of the ligand, in this case Λ-DPPZ, into buffer and the dotted line is the titration of the identical sample into an excess concentration of DNA. When integrating peaks to obtain enthalpy values, it was necessary to exclude data from the first two additions of ligand because these injections of titrant are not accurately dispensed due to diffusion of the titrant in the syringe needle during the thermal equilibration period. The negative deflections shown in Figure 5 correspond to *positive* enthalpies, because of the conventions adopted in the design of the Hart Scientific instrument used for these experiments. Both Δ- and Λ-DPPZ and racemic $[Ru(phen)_3]^{2+}$ gave nonnegligible heats of dilution when titrated into buffer. The averages of 20−25 determinations of dilution heats were as follows: Δ-DPPZ, +220 (±20) cal mol^{-1}; Λ-DPPZ, +160 (±20) cal mol^{-1}; $[Ru(phen)_3]^{2+}$, +150 (±20) cal mol^{-1}. Binding enthalpies obtained for Δ- and Λ-DPPZ and for racemic $Ru(phen)_3$, after having been corrected for these heats of dilution of the ligand, are shown in Table 1, along with ΔH values for ethidium and daunomycin for comparison. All the ruthenium compounds bind endothermically to calf thymus DNA, in sharp contrast to the proven intercalators which have relatively large negative enthalpies.

The calorimetric determination of the enthalpies of binding of the Ru compounds was difficult because of their small magnitude. The *sign* of the enthalpy is unambiguously determined, but the error in the ΔH values we have determined is larger than we would prefer (no better than 10−20%). The relatively large error is due, in part, to the propagation of error that results from the need to correct the heat of DNA binding for the heat of dilution of the ligand. Both quantities have error, and the propagated error is larger than that of either quantity alone.

The positive sign of ΔH for Δ- and Λ-DPPZ was confirmed using thermal difference spectra, using the method fully described before[9a] (data not shown). In such experiments, the effect of temperature on binding was examined by recording the visible absorbance spectra of the bound ligand at a reference temperature of 25 °C, and then at 10 and 45 °C. For both Δ-

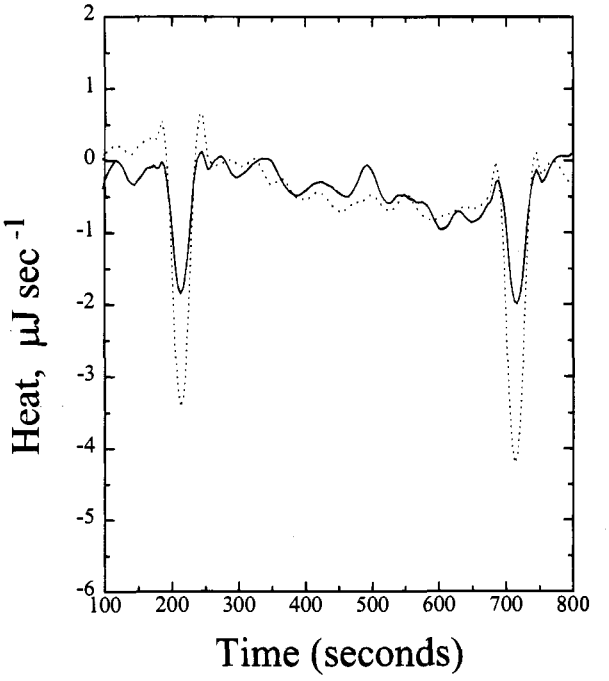

Figure 5. Sample primary calorimetric data from the titration of Λ-$[Ru(phen)_2DPPZ]^{2+}$ into Tris−HCl, 50 mM NaCl buffer (dotted line) and into calf thymus DNA (solid line) at 25 °C. Each peak represents the heat produced by the injection of a 5 μL aliquot of drug into either buffer (heat of dilution) or DNA (heat of formation of DNA/drug complex). During each experiment a total 75 μL of drug was injected into the titrate to give a total of 15 determinations per titration. Enthalpy values were obtained by integration of the peaks using software from Hart Scientific.

Table 1. A Comparison of Thermodynamic Parameters[a] for Ligand Binding to DNA

compd	$K_{obs}/10^4$	ΔG_{obs}	**SK**	ΔG_{pe}	ΔG_t	ΔH	ΔS
Δ-DPPZ	320	−8.9	1.9	−3.3	−5.6	$+0.2_5$	+30.8
Λ-DPPZ	170	−8.5	2.1	−3.7	−4.8	+2.9	+38.1
ethidium[b]	125	−8.3	1.1	−1.9	−5.8	−8.8	−1.4
daunomycin[c]	643	−9.1	1.2_5	−2.2	−6.9	−10.4	−4.7
Δ-Ru(phen)$_3$[d]	0.9_7	−5.4	1.4	−2.4	−3.1	+2.6*	+26.8
Λ-Ru(phen)$_3$[d]	1.1	−5.5	1.2	−2.2	−3.4		

[a] K_{obs} ($M(bp)^{-1}$) is the binding constant for the interaction of a ligand molecule with DNA and refers to solutions containing 0.05 M NaCl at 20 °C. ΔG_{obs} (kcal mol^{-1}) is the binding free energy calculated from the equation $\Delta G_{obs} = -RT\ln K_{obs}$. The parameter **SK** is the slope of the graphs shown in Figure 4. ΔG_{pe} and ΔG_t (kcal mol^{-1}) are respectively the polyelectrolyte and the "nonelectrostatic" contributions to the binding free energy. The polyelectrolyte contribution was calculated from the equation, $\Delta G_{pe} = \mathbf{SK}RT\ln[Na^+]$, evaluated at $[Na^+]$ = 0.05 M. The "nonelectrostatic" portion of the free energy was calculated by difference. The enthalpy values for Δ- and Λ-DPPZ and Ru(phen)$_3$ were determined calorimetrically and then used to determine $\Delta S°$ by difference. [b] Data for K_{obs}, ΔG_{obs}, ΔH, and ΔS were taken from ref 26. The values for **SK**, ΔG_{pe}, and ΔG_t were calculated from ref 27. [c] Data taken from Chaires et al.[28] [d] Data from Satyanarayana et al.,[9b] the value for enthalpy (*) refers to the racemic mixture of Δ- and Λ-Ru(phen)$_3$.

and Λ-DPPZ the spectra obtained at 45 °C showed a *decrease* in absorbance at 439 nm, relative to spectra recorded at 25 °C. This reduced absorbance corresponds to *increased* binding. The converse is true at 10 °C, where there is an *increase* in absorbance at 439 nm, indicating a *decrease* in the amount of bound ligand. While the magnitude of the spectral differences was small, typically about 0.005 absorbance units, the observed difference spectra were well above the noise level of the instrument, and were highly reproducible. These changes in absorbance at λ_{max} that accompany changes in temperature show that the binding enthalpies for these compounds are positive in sign and small in magnitude, confirming the results obtained by isothermal calorimetry.

The experimentally determined value for ΔH allows the calculation of the entropy from the following relationship:

$$\Delta S = (\Delta H - \Delta G°)/T$$

Comparative values of the entropy for the ruthenium compounds and proven intercalators are summarized in Table 1.

Viscosity Measurements. The mode of binding of Δ- and Λ-DPPZ was investigated using viscosity measurements. Figure 6 shows that increasing amounts of ligand increase the relative viscosity of rod-like calf thymus DNA. Such an increase is one critical test for classical intercalation as described by Lerman.[23] The observed increase in relative viscosity occurs as a result of a length increase of the duplex following intercalation. Since the *maximum* drug to base pair ratio in these viscosity experiments was 0.3, the length increase results from the process with the 1:3 stoichiometry, which we therefore conclude corresponds to an intercalative mode of binding.

Fluorescence Energy Transfer Experiments. Figure 7 shows the results of fluorescence energy transfer experiments[20-22] designed to test the binding mode of [Ru(phen)$_2$DPPZ]$^{2+}$ and [Ru(phen)$_3$]$^{2+}$. Energy may be transferred from the DNA bases to excite an intercalated fluorophore. No such energy transfer is possible for a groove or surface bound fluorophore, since both the transfer distance and the orientation of the donor–acceptor dipoles are unfavorable. Both isomers of RuDPPZ are excited by absorbance by DNA bases (Figure 7), providing strong evidence for an intercalative binding mode. Since these experiments were conducted with a large molar excess of DNA,

(23) Lerman, L. S. *J. Mol. Biol.* **1961**, *3*, 18–30.

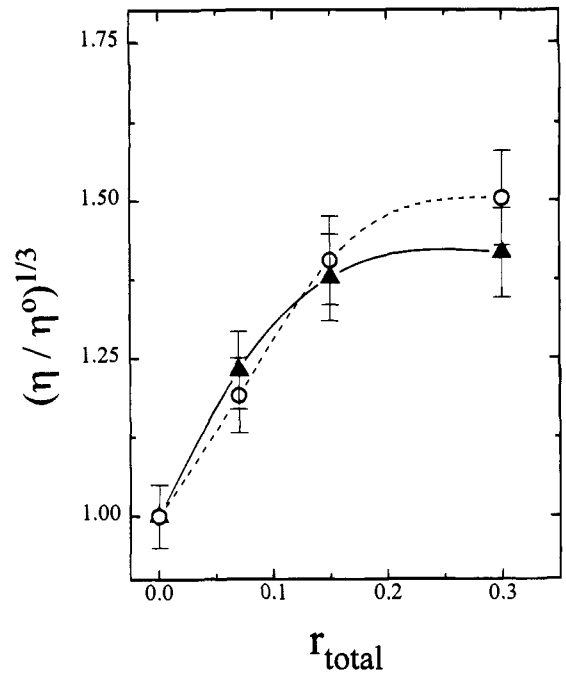

Figure 6. The effect of the addition of increasing amounts of Δ-DPPZ (circles) and Λ-DPPZ (triangles) on the specific relative viscosity of calf thymus DNA. The total ligand to base pair ratios measured were 0.07, 0.15, and 0.3.

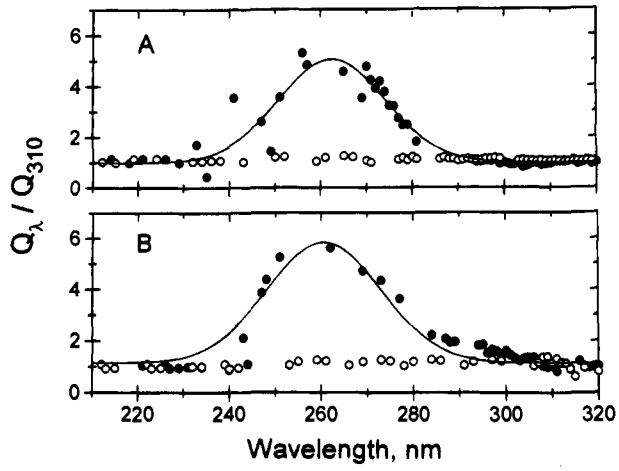

Figure 7. Fluorescence energy transfer from DNA to bound [Ru(phen)$_2$DPPZ]$^{2+}$. The relative fluorescence quantum yield of bound versus free ligand is shown as a function of excitation wavelength. (A) The Λ isomers of [Ru(phen)$_2$DPPZ]$^{2+}$ (filled circles) or [Ru(phen)$_3$]$^{2+}$ (open circles). (B) The Δ isomers of [Ru(phen)$_2$DPPZ]$^{2+}$ (filled circles) or [Ru(phen)$_3$]$^{2+}$ (open circles).

these results would apply to the binding process with the 1:3 drug to base pair stoichiometry. Experiments under similar conditions show no energy transfer between DNA bases and Ru(phen)$_3$, indicating that no intercalation occurs.

Discussion

The interaction of transition metal complexes with DNA is an area of intense current interest, in part because of the potential of these compounds as novel probes of DNA structure.[8] While considerable attention has been given to the photophysical properties of transition metal complexes bound to DNA, and to the possible structures of such complexes, little is known about the energetics of their binding to DNA. Thermodynamic studies are a necessary compliment to structural studies and are essential for a complete understanding of the molecular forces

that drive a particular binding interaction. By using a combination of spectroscopic and calorimetric titration methods, complete thermodynamic profiles (ΔG, ΔH, and ΔS) for the interaction of the Δ and Λ isomers of $[Ru(phen)_2DPPZ]^{2+}$ and of racemic $Ru(phen)_3$ with DNA have been obtained and are described here. A novel and surprising result to emerge from these studies is that the binding of all of these transition metal complexes to DNA is *entropically driven*.

Binding Stoichiometry. The aim of equilibrium binding studies is to determine for the ligand–macromolecule interaction under study how many ligands bind and how tightly they are associated. The optimal experimental conditions for the determination of each of these facets are usually mutually incompatible. Determination of binding stoichiometry requires high reactant concentrations to ensure complete binding of all available ligand and saturation of all available sites. Determination of binding equilibrium constants requires, in contrast, reactant concentrations that are approximately equal to the reciprocal of the ligand association constant, typically in the μM range for small DNA binding agents. The method of continuous variations[14] is optimal for defining binding stoichiometry. The "Job plot" presented in Figure 2 reveals two distinct binding stoichiometries for both the Δ and Λ isomers of $[Ru(phen)_2DPPZ]^{2+}$. Each compound shows one complex corresponding to a 1:3 ligand-to-DNA bp ratio. A second complex is observed that corresponds to more than 1 ligand bound per DNA base pair. The latter process is weaker than the former, and appears only at high reactant concentrations. Hiort *et al.*[12] reported a second, low-affinity, binding mode based on luminescence titration experiments, consistent with the second binding mode observed in continuous variations experiments reported here. In all subsequent experiments described here, conditions were chosen to selectively study the higher affinity, 1:3 binding mode.

Binding Mode. In the absence of high-resolution structural data, hydrodynamic methods that are sensitive to DNA length changes are arguably the most critical tests of the classical intercalation model, and therefore offer the most definitive means of inferring the binding mode of DNA binding agents. Both the Δ and Λ isomers of $[Ru(phen)_2DPPZ]^{2+}$ increase the relative viscosity of rod-like DNA in a manner consistent with the behavior expected from classical intercalation (Figure 6). Such behavior is in sharp contrast with the behavior shown by $Ru(phen)_3$ isomers, neither of which increased DNA viscosity, and neither of which intercalate into DNA.[9] We conclude from viscosity studies that both Δ- and Λ-$[Ru(phen)_2DPPZ]^{2+}$ intercalate into DNA. Since viscosity experiments were conducted at molar ratios of drug to base pair of 0.3 or less, we conclude that the process with the 1:3 stoichiometry apparent in Job plots (Figure 2) is an intercalation event. We have no experimental data to assign a binding mode to the second process with a stoichiometry of greater than 1 ligand per base pair, but we speculate that it would correspond to binding of the ligand on the DNA surface.

Strong additional support for intercalative binding of Δ- and Λ-RuDPPZ is provided by fluorescence energy transfer experiments (Figure 7). Energy transfer between DNA bases and a bound fluorophore is only possible if the fluorophore is intercalated, and stacked among the base pairs. Comparative studies of the proven intercalator ethidium and the known groove binding agent Hoechst 33258 have directly demonstrated that fluorescence energy transfer experiments can reliably distinguish between intercalated and groove bound fluorophores.[22] Neither isomer of $Ru(phen)_3$ shows fluorescence energy transfer when bound to DNA, from which we conclude that neither is intercalated into DNA. That conclusion is consistent with viscosity results previously reported.[9]

Additional support for intercalative binding of $Ru(phen)_2DPPZ$ comes from published flow linear dichroism studies.[12] A quantitative analysis of linear dichroism spectra published for Δ and Λ enantiomers of $Ru(phen)_2DPPZ$[12] shows that in both cases the plane of the DPPZ ligand is perpendicular to the DNA helix axis (within 10°), as expected for an intercalative binding geometry (Lincoln and Norden, unpublished data). Racemic $[Ru(phen)_2DPPZ]^{2+}$ was proposed to bind to DNA by intercalation, based on the results of a DNA unwinding assay, and a variety of luminescence measurements.[3]

Binding Affinity. Fluorescence titrations (Figure 3) were conducted at fixed ligand concentrations, under conditions designed to accurately measure the binding constant for the highest affinity binding mode. Further, these conditions allow neglect of possible neighbor exclusion effects. For both the Δ and Λ isomers, the midpoint of the titration curve is dependent upon the total ligand concentration, a result that is expected if the total ligand concentration exceeds the reciprocal of the association constant. Fits of the data of Figure 3 yield estimates of $3.2\ (\pm 0.5) \times 10^6$ M $(bp)^{-1}$ and $1.7\ (\pm 0.2) \times 10^6$ M $(bp)^{-1}$ for the association of the Δ and Λ isomers of $[Ru(phen)_2DPPZ]^{2+}$, respectively, with calf thymus DNA in 50 mM NaCl. Previous estimates for these binding constants, obtained in solutions containing 10 mM NaCl, were found to be approximately 6×10^7 M $(site)^{-1}$.[12] By using the salt dependence of the binding constant determined in Figure 4 and the binding constants determined in 50 mM NaCl, an estimate of $K = 5.7 \times 10^7$ M $(bp)^{-1}$ at 10 mM NaCl may be calculated from our data. This estimate is in excellent agreement with the previously published value.[12] Binding of $[Ru(phen)_2DPPZ]^{2+}$ to DNA is thus very tight. There appears to be only a slight enantioselectivity, with the Δ isomer binding only slightly more tightly to DNA than does the Λ isomer. Binding free energies may be calculated from these estimates of the binding constant by using the standard relation $\Delta G^\circ = -RT\ln K$, yielding the values shown in Table 1.

Salt Dependence of Binding. Because $[Ru(phen)_2DPPZ]^{2+}$ is a dication, its binding to DNA is thermodynamically linked to Na^+ binding to DNA, and as a result, its DNA binding constant will depend on the total Na^+ concentration. Polyelectrolyte theories based on Manning's counterion condensation model provide a description of the process[17,24] and a basis for interpreting the data of Figure 4. For both isomers of $[Ru(phen)_2DPPZ]^{2+}$, the slope $(\delta \log K/\delta \log[Na^+])$ is found to be near 2. A slope of 1.76 is predicted by Record *et al.*,[17] but a higher value of 2.24 is predicted for an intercalator by Friedman and Manning.[24] The latter includes an additional contribution to counterion release arising from increased phosphate spacing resulting from intercalation. The values we observe for both Δ- and Λ-$[Ru(phen)_2DPPZ]^{2+}$ are in very good agreement with these theoretical predictions. For the Δ isomer, we observe nonlinear behavior for $[Na^+] > 0.125$. While we can offer no detailed explanation for such behavior, we have found it to be reproducible. The original polyelectrolyte theories of both Manning and Record are limiting laws, and they are strictly applicable only in dilute concentrations of all ionic species. These theories predict linear slopes for $(\delta \log K/\delta \log[Na^+])$ only when changes in protonation, hydration, and anion binding of both the ligand and macromolecule are negligible. Given these considerations, the nonlinearity apparent in Figure 4 at the higher salt concentrations is not surprising, and it is not deemed to be

(24) Friedman, R. A. G.; Manning, G. *Biopolymers* **1984**, *23*, 2671–2714.

cause for serious concern. Pronounced nonlinearity in log K *versus* log M^+ plots for protein–DNA interactions was recently described and discussed in detail.[25]

Calorimetric Enthalpy Values for [Ru(phen)$_2$DPPZ]$^{2+}$ Binding to DNA. Microtitration calorimetry offers the most direct means of measuring DNA binding enthalpies. The surprising result to emerge from such studies is that for both Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$, DNA binding is characterized by a *positive* enthalpy (Figure 5, Table 1). Binding of Ru(phen)$_3$ to DNA is also characterized by a positive enthalpy. While the error in our best estimates of enthalpy values is larger than we would like, probably no better than 10–20%, we note that this system presents substantial difficulties. Small enthalpy values are difficult to measure accurately, even by direct titration calorimetry. Further, the error increases because of the propagated error introduced by the correction for the heat of dilution of the Ru compounds. The positive sign of the enthalpy values is unambiguously determined by calorimetry and has been confirmed by independent spectroscopic studies. The positive enthalpy values for the DNA binding of [Ru(phen)$_2$DPPZ]$^{2+}$ isomers are unusual in comparison to values normally found for intercalators. Our best estimates for binding enthalpy values are collected in Table 1.

Dissecting the Free Energy of [Ru(phen)$_2$DPPZ]$^{2+}$ Binding to DNA. The combined results of binding and calorimetric studies allow us to construct a complete thermodynamic profile for the binding of [Ru(phen)$_2$DPPZ]$^{2+}$ and Ru(phen)$_3$ to DNA and to begin to dissect the observed binding free energy into its component parts. From the dependence of the binding constant on salt concentration, the observed binding free energy may be partitioned into two contributions:

$$\Delta G^\circ_{obs} = -RT\ln K = \Delta G_t + \Delta G_{pe}$$

where ΔG_t is the "nonelectrostatic" contribution to the binding free energy and ΔG_{pe} is the "polyelectrolyte" contribution. The latter term may be calculated from the experimentally determined quantity $(\delta \log K/\delta \log[Na^+]) = \mathbf{SK}$. Record and coworkers[17] have shown that $\Delta G_{pe} = (\mathbf{SK})RT\ln[MX]$, where MX is the monovalent salt concentration. Table 1 summarizes the comparative energetics for the binding of proven intercalators, ethidium and daunomycin, Δ- and Λ-DPPZ and racemic Ru(phen)$_3$. The magnitude of ΔG_t provides a measure of the nonelectrostatic forces (hydrogen bonding, van der Waals interactions) that stablize the DNA/ligand complex. ΔG_{pe} is the free energy contribution arising from coupled polyelectrolyte effects, the most important of which is the release of condensed counterions from the DNA helix upon binding of the charged ligand. Several important features emerge from these calculations. ΔG_t for ethidium and daunomycin is large in magnitude, indicating that nonelectrostatic forces play a significant role in the stabilization of their DNA complexes. For both isomers of [Ru(phen)$_2$DPPZ]$^{2+}$, the value of ΔG_t is also substantial, indicating that nonelectrostatic forces play a major role in stabilizing the ligand–DNA complex. The magnitudes of ΔG_t for both enantiomers of [Ru(phen)$_2$DPPZ]$^{2+}$ are about 2 kcal mol^{-1} larger than the ΔG_t values observed for Ru(phen)$_3$ binding to DNA. This would be consistent with intercalative binding by Δ- and Λ-DPPZ, with concomitant stacking interactions with the DNA base pairs, while [Ru(phen)$_3$] does not intercalate, and would consequently have less favorable stacking interactions. It is of interest that ΔG_t for Δ- and Λ-[Ru(phen)$_2$DPPZ]$^{2+}$ is of comparable magnitude to the value observed for ethidium, the prototypical intercalator, but is slightly less than the value found for daunomycin, which not only intercalates but is also stabilized by several specific hydrogen bonds between the drug and DNA base pairs. The relative contributions to ΔG_{obs} for several DNA binding agents are shown in Figure 8. For daunomycin, binding is largely nonelectrostatic, while for ethidium and Δ- and Λ-DPPZ, ΔG_t comprises a substantial portion of the total binding free energy. The tighter DNA binding of Δ- and Λ-DPPZ relative to ethidium arises from a greater contribution from ΔG_{pe}.

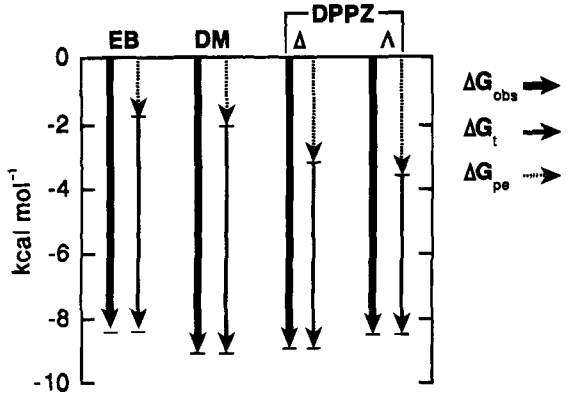

Figure 8. The dissection of the binding free energy (thick solid line) into its polyelectrolyte (dotted line) and nonelectrostatic (solid line) contributions for a series of DNA binding ligands. The binding free energies and computed polyelectrolyte free energy contribution refer to solutions containing 50 mM NaCl.

Direct determination of the enthalpy of binding by titration calorimetry allows the observed binding free energy to be dissected into its energetic and entropic components, using the standard relation $\Delta G = \Delta H - T\Delta S$. *Positive* values of ΔH are observed for both DPPZ enantiomers, indicating that the favorable binding free energy is derived from the positive entropy for the binding process, and the resultant large, negative $T\Delta S$ term. Inspection of Table 1 shows that the DNA binding of the proven intercalators ethidium and daunomycin is driven by a large negative enthalpy value, and that their binding is *opposed* by a positive $T\Delta S$ term. The DNA binding of the Δ and Λ isomers of [Ru(phen)$_2$DPPZ] is entirely *entropically* driven, an unusual finding for intercalating agents. Figure 9 summarizes the enthalpic and entropic contributions to the DNA binding free energy for [Ru(phen)$_2$DPPZ] isomers and several other DNA binding agents.

Comparison of DNA Binding Thermodynamics. The entropically driven DNA binding of the Δ and Λ isomers of [Ru(phen)$_2$DPPZ] is unlike the thermodynamics of DNA binding of proven intercalators like ethidium and daunomycin. However, actinomycin, another intercalator, is reported to bind to DNA with an enthalpy near zero.[29] Its DNA binding must therefore also be entropically driven. Actinomycin shares with [Ru(phen)$_2$DPPZ] an interesting structural feature. Both compounds are comprised of an intercalating chromophore, to which bulky constituents are attached. In the case of actinomycin, these are cyclic peptides, while in the case of [Ru(phen)$_2$DPPZ], these are the phenanthroline wings. Fitting these bulky groups into the DNA grooves may result in similar energetic costs for both actinomycin and [Ru(phen)$_2$DPPZ] and may yield a

(25) Stickle, D. F.; Liu, G.; Fried, M. G. *Eur. J. Biochem.* **1994**, *226*, 869–876.
(26) Hopkins, H. P.; Fumero, J.; Wilson, W. D. *Biopolymers* **1990**, *29*, 445–459.
(27) Hopkins, H. P.; Wilson, W. D. *Biopolymers* **1987**, *26*, 1347–1355.
(28) Chaires, J. B.; Priebe, W.; Graves, D. E.; Burke, T. G. *J. Am. Chem. Soc.* **1993**, *115*, 5360–5364.
(29) (a) Gellert, M.; Smith, C.; Neville, D.; Felsenfeld, G. *J. Mol. Biol.* **1965**, *11*, 445–457. (b) Crothers, D. M.; Sabol, S.; Ratner, D. L.; Mueller, W. *Biochemistry* **1968**, *7*, 1817–1822. (c) Crothers, D. M.; Ratner, D. L.; Mueller, W. *Biochemistry* **1968**, *7*, 1823–1827.

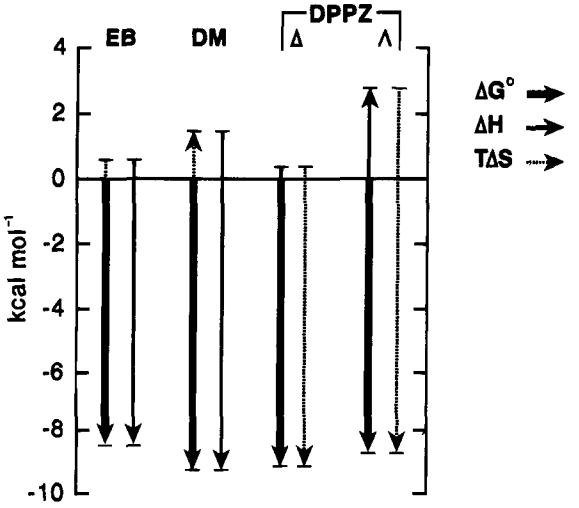

Figure 9. A comparison of the enthalpic and entropic contributions to the binding free energy for a series of ligands binding to DNA. Data are shown for ethidium bromide (EB), daunomycin (DM), and Δ- and Λ-DPPZ. This diagram is a graphical representation of the relationship, $\Delta G^\circ = \Delta H - T\Delta S$. For the proven intercalators, ethidium and daunomycin, $-T\Delta S$ is small and positive, and ΔH is large and negative, hence binding is largely enthalpically driven. For the ruthenium complexes the converse is true, and binding is entropically driven.

common pattern to their thermodynamic profiles. Ethidium and daunomycin, in contrast, have small constituents (a phenyl group and an amino sugar, respectively) that can readily fit into the minor groove of DNA.

Molecular Interpretation of Thermodynamic Quantities. Examination of the relative magnitudes of the thermodynamic parameters characteristic of the binding of DPPZ enantiomers to DNA allows an assessment of the molecular interactions that stabilize the binding complex. Hydrogen bonding and van der Waals interactions are generally characterized by negative standard enthalpies and entropies of interaction. Electrostatic interactions generally exhibit small enthalpy changes and positive entropy values. "Hydrophobic" interactions are generally characterized by positive enthalpy and entropy changes and by negative heat capacity changes. The favorable binding free energy of both isomers of $[Ru(phen)_2DPPZ]$ arises from a large $T\Delta S$ term. We note, first, that the pattern of the thermodynamic profile of $[Ru(phen)_2DPPZ]$ DNA binding (positive enthalpy, positive entropy) is typical of that observed for "hydrophobic" interactions,[30] in which nonpolar groups are buried and rendered inaccessible to solvent. Intercalation of the DPPZ moiety certainly would be consistent with such a phenomenon. However, stacking interaction, as would be expected to form between the DNA bases and the intercalated DPPZ, ought to result in a negative enthalpy term. Evidently, in this particular case, such stacking interactions are energetically balanced and overcome by other types of molecular interactions. Over one-third of the observed binding free energy may be attributed to ΔG_{pe}, the polyelectrolyte contribution. This term arises primarily from condensed counterion release, a process that is believed to be nearly entirely entropic.[17] This process is surely a major contributor to the overall entropy that drives the binding reaction. To illustrate the contribution, the the Δ isomer, $T\Delta S$ may be calculated to be -11.1 kcal mol^{-1} at 20 °C. ΔG_{pe} for this isomer is -3.7 kcal mol^{-1} (Table 1), a value that equals about one-third of the total entropic contribution. An additional entropic contribution might come from changes in hydration of both the ligand and DNA. Disruption of bound water would be expected to contribute an unfavorable positive enthalpy but a favorable positive entropy. The signs of ΔH and ΔS are consistent with the removal of water from either the ligand or DNA or both upon binding. We conclude that the entropically driven binding of Δ- and Λ-$[Ru(phen)_2DPPZ]$ to DNA results primarily from counterion release, changes in hydration, and "hydrophobic" interactions resulting from the transfer of the DPPZ moiety from the aqueous solvent into the interior of the DNA helix.

(30) (a) Kauzman, W. *Adv. Protein Chem.* **1959**, *14*, 1–63. (b) Eftink, M. R.; Biltonen, R. L. In *Biological Microcalorimetry*; Academic Press, Inc.: New York, 1980; pp 343–412.

Summary

Fluorescence titration methods and isothermal titration calorimetry have been used to obtain complete thermodynamic profiles (ΔG°, ΔH, ΔS) for the interaction of the Δ and Λ enantiomers of $[Ru(phen)_2DDPZ]^{2+}$ with DNA. The results show, surprisingly, that binding of both enantiomers to DNA is entirely entropically driven. Both enantiomers intercalate into DNA, as judged from the results of relative viscosity experiments and fluorescence energy transfer experiments. The thermodynamics of their DNA binding, however, is unlike those observed for the proven intercalators ethidium and daunomycin. The pattern of the thermodynamic profile of $[Ru(phen)_2DDPZ]^{2+}$ binding to DNA suggests that the binding reaction is driven primarily by hydrophobic interactions, hydration changes, and polyelectrolyte effects resulting from the release of condensed counterions.

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