Interactions of Tris(phenanthroline)ruthenium(II) Enantiomers with DNA: Effects on Helix Flexibility Studied by the Electrophoretic Behavior of Reptating DNA in Agarose Gel†

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A combination of measurement of mobility and orientational dynamics of long reptating DNA in agarose gel has been used to reveal how the binding of Δ and Λ enantiomers of the tris(phenanthroline)ruthenium(II) ion affects the flexibility of the DNA helix. The mobility data, and data over the step length and period time of the reptation cycle, obtained in the presence of the respective enantiomer, are compared with those of free DNA and with data obtained earlier on DNAs with known variations in helix flexibility. The results suggest that the Δ form induces kinks in the DNA helix while the Λ form gives rise to a local stiffening of the helix.

1. Introduction

Metal ion complexes of polypyridyl ligands have been extensively studied for many years as probes of DNA structure and function and in the hope of developing new therapeutic agents. A particular attraction of several of these metal complexes is that they exist as chiral molecules with the possibility of stereoselective binding.[1] One of the most well-studied members in this group is tris(phenanthroline)ruthenium(II) ($[Ru(phen)_3]^{2+}$). Many experimental techniques have been applied to study the interaction of this compound with DNA, but despite this its binding mode and its effect on the DNA structure are uncertain and have been the subject of much controversy (for recent reviews see refs 2 and 3). Unwinding studies with closed circular DNA and absorption and fluorescence spectroscopy data were proposed to evidence that both the Δ and the Λ enantiomer of $[Ru(phen)_3]^{2+}$ bind to DNA by intercalation.[4,5] From 1-D NMR it was first inferred that Δ intercalates and that Λ binds in the minor groove[6] while 2-D NMR later indicated minor groove contacts, but no proper intercalation, for both enantiomers.[7] From linear dichroism and equilibrium and viscosity experiments it had been concluded that neither Δ nor Λ is bound to the DNA by intercalation.[8,9] This view was also supported by a scanning force microscopy study, which revealed that neither enantiomer causes any change in the length of DNA (intercalating ligands necessarily increase the contour length of DNA).[2] However, a recent reevaluation of linear dichroism data for Δ and Λ showed their angular binding geometries to be consistent with intercalation and further that one of the phenanthrolines of each enantiomer has a limited stacking interaction with the DNA bases.[3] To reconciliate the apparently contradictory findings, it was proposed that Δ and Λ bind to a nonclassical intercalation pocket, formed either by an opening wedged between two base pairs (semi-intercalation) or by the indenture of one base pair toward the opposite groove (quasi-intercalation).[3]

Since the geometry of the intercalation pocket can be anticipated to have a profound influence on the hydrodynamic properties of DNA, it is interesting to note that the two enantiomers vary in their effects. Flow linear dichroism data show that already at small binding ratios Δ drastically reduces the orientation of DNA while the Λ isomer has no significant effect on the orientation.[8] The decreased orientability with Δ is consistent with a reduced persistence length, but the mechanism is unknown; flexible points or static kinks were proposed. Results from viscosity experiments on rodlike DNA show that Λ has little effect on the hydrodynamic length but indicates that Δ may kink DNA, which suggests a semi-intercalative binding mode for this enantiomer.[9]

In polyacrylamide gel electrophoresis linear DNA molecules containing regions of curvature have reduced mobility, a fact that has been widely used to detect and localize both intrinsically bent sequences and ligand-induced bends.[10] An important factor for the sensitivity of detection of DNA curvature variation in this gel is clearly the fact that the gel pore sizes are much smaller than the persistence length of DNA.[11] However, in a study where we compared mobilities of linear DNA in polyacrylamide gels in the presence of the Δ or Λ enantiomer of $[Ru(phen)_3]^{2+}$, no significant difference in the mobilities was obtained (unpublished results). In gels with pores larger than those of polyacryamide gels, such as agarose gels, small curved DNA fragments show normal migration properties. The pore sizes in agarose gel are, however, of the same order as the persistence length of DNA[12] and for a long reptating (see section 2.5 below) DNA, chain flexibility should be important for the electrophoretic behavior, as has also recently been shown by us.[13] Kinks, flexible bends, and flexible joints in the DNA helix, induced by binding Pt compounds to the DNA, were found to have different effects on the average step length and average period time (the ratio of which quantities is equal to the electrophoretic velocity) of the reptation cycle. The results suggested that a combination of mobility and orientation measurements can be utilized for distinguishing different kinds of structural alterations in the DNA, which motivated us to perform the study presented here on DNAs complexed with the two enantiomers of $[Ru(phen)_3]^{2+}$. The experimental results are discussed and interpreted in terms of variations in helix flexibility caused by the binding of the metal complex.

† Nonstandard abbreviations: Δ, Δ enantiomer of tris(phenanthroline)-ruthenium(II); Λ, Λ enantiomer of tris(phenanthroline)ruthenium(II).

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2. Materials and Methods

2.1. Chemicals and Sample Preparation. T2 DNA (164 kbp) and T7 DNA (38 kbp) were obtained from Sigma, and λ-DNA Hind III digest (2027−23130 bp) was from Pharmacia. To break the annealed sticky end that forms between the 4 kbp and 23 kbp fragments, the digests were heated to 65 °C for 5 min and quickly cooled on ice before gel loading. The $[Ru(phen)_3]^{2+}$ racemate was synthesized and resolved into optical isomers according to the method described by Dwyer and Gyarfas.[14] CD and absorption measurements were used to ensure chemical and enantiomeric purity (>99%). The concentration of $[Ru(phen)_3]^{2+}$ in the pure solutions was determined from absorbance at 447 nm by using the molar absorptivity $\epsilon_{447} = 19\,000\ M^{-1}\ cm^{-1}$.[15] Ultrapure DNA grade agarose was from BioRad, ethidium bromide (EtBr) was from Sigma, and all other chemicals were of analytic reagent grade.

All experiments were carried out in 1% (w/w) agarose gels. The agarose was dissolved in electrophoresis buffer (50 mM Tris base and 50 mM boric acid) by boiling. In the experiments where the effect of $[Ru(phen)_3]^{2+}$ on DNA was to be studied the gel solutions were allowed to cool to 40 °C before the complex was added (to a final concentration of 9 μM) and carefully mixed with these (to secure a uniform distribution of the complex throughout the gels). The solutions were then poured into a gel tray divided by spacers to get gel strips for mobility measurements[16] or into a vertical cell for orientational measurements.[17] The gels were allowed to solidify at room temperature for at least 1 h. For mobility experiments 0.5 μg of DNA was loaded in each well, and for LD measurements 10 μg.

Agarose matrices usually contain some anionic sulfate and pyruvate groups, which induce electroosmotic flow toward the cathode.[18] The possibility that the metal complex is adsorbed to the gel was therefore checked since adsorption of charged species to the gel fibers could change the flow and thus the electrophoretic behavior. Binding of the metal complex to the gel may also reduce the DNA−gel repulsion, which will increase the apparent pore size of the gel. These effects, and if the enantiomers are adsorbed to different extents (agarose is chiral), could give rise to artificial differences in the apparent DNA behavior during electrophoresis. Metal complex solutions of various concentrations (0.1−15 μM) were therefore equilibrated with pieces of gel (volume ratio 3:1 solution:gel) and after 24 h the metal complexes had redistributed between solution and gel. However, within experimental error (1%) the decrease in solution concentration corresponded to the dilution effect of the added gel volume and no significant difference was noticed between the enantiomeric forms of the complex.

2.2. Electrophoretic Mobility. The mobility measurements were performed at field strengths between 5 and 17 V/cm in constant field agarose gel electrophoresis in a Mini Sub cell from BioRad. The buffer covering the gel had the same composition as that used for the preparation of the gel (50 mM Tris base and 50 mM boric acid with or without 9 μM $[Ru(phen)_3]^{2+}$). In the experiments with $[Ru(phen)_3]^{2+}$ the concentration of the metal complex in this buffer remained constant during the electrophoresis, as determined from absorbance measurements at 447 nm. A corresponding determination of the $[Ru(phen)_3]^{2+}$ concentration in the gel has not been possible due to the turbidity of the gel but it is reasonable to assume that it has also been constant and equal to that in the overlayered solution. In all runs the temperature has been kept at 20 ± 2 °C by circulating the electrophoresis buffer through an outer heat exchanger in a thermostating bath. The circulation also secured a constant concentration of the buffer and the metal complex along the cell.

After electrophoresis the gels with native DNA were directly stained with EtBr and then destained in water prior to the gel being photographed. When $[Ru(phen)_3]^{2+}$ was present, the gels had to be washed in buffer before they were stained with EtBr, since the presence of the metal complex prevented the staining of the DNA. Presented data are averages of measurements on at least three gels.

2.3. Linear Dichroism. Linear dichroism (LD) spectroscopy has been used to follow the orientational behavior of the DNA during the electrophoresis. LD is defined as the difference in the absorption of light polarized parallel and perpendicular to a given laboratory axis through the sample, in our case the electrophoresis direction.

$$LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda) \tag{1}$$

where λ denotes the wavelength of light. On a molecular level the absorption process is determined by a vectorial property known as the transition dipole moment, the absorption being maximum when the electric vector of light is polarized parallel to the transition moment and zero when perpendicular to it. LD can therefore provide information about molecular orientation when the transition moments are known. In the absence of orientation in the sample $A_{\parallel} = A_{\perp}$ and LD = 0. If the molecules are oriented, the orientation can be characterized by the reduced linear dichroism (LD^r) defined as[19]

$$LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) \tag{2}$$

where A_{iso} denotes the absorbance of the corresponding isotropic sample. The LD^r factorizes into a product between an optical factor, O, and an orientation factor, S.[19]

$$LD^r(\lambda) = SO \tag{3}$$

O is related to the effective angle between the transitions moments contributing at the wavelength λ and the principal orientation axis of the molecule (in our case the DNA helix axis), whereas S describes the average orientation of this axis relative to the reference direction. S is 0 in a sample where the molecules are randomly oriented and 1 in a sample where they are perfectly oriented parallel to the reference direction.

With the conditions prevailing in our experiments the double-stranded DNA has been in its B-form conformation.[20] For pure DNA S can then be determined directly from eq 3 by measuring LD^r of the base absorption band at 260 nm and by using an optical factor of −1.48.[19] However, with $[Ru(phen)_3]^{2+}$ bound to DNA S cannot be determined since the $[Ru(phen)_3]^{2+}$ transitions in the UV region overlap the DNA transitions and the high background absorption from the free metal complex makes a reliable determination of A_{iso} impossible. It has thus not been possible to quantify the effect of $[Ru(phen)_3]^{2+}$ on the degree of orientation but only on the orientational dynamics, which in this work is directly given by the time behavior of the LD since the change in A_{iso} during the relatively short electrophoretic pulses applied in the experiments can be expected to be very small.

2.4. Linear Dichroism Measurements. *2.4.1. Flow Orientation.* LD spectra of flow-oriented T2-DNA and T2-DNA in the presence of $Ru(phen)_3]^{2+}$ were obtained using a Couette-device described in ref 19.

2.4.2. Electrophoretic Orientation. LD experiments were performed on T2 DNA and T2 DNA in the presence of

$[Ru(phen)_3]^{2+}$ at a gel concentration (1%) and a temperature (20 ± 2 °C) equal to that in the mobility experiments. The electrophoresis cell, the optical equipment, and the technique used for the spectroscopic measurements have been described in detail earlier.[17] The DNA was migrated to the measuring position in the gel by a constant field (2.5 V/cm, 10 h). LD of the DNA sample was measured during square-formed voltage pulses. The measurements on the native DNA sample were performed in the absorption band of the DNA bases (260 nm), whereas the measurements on the DNA−$[Ru(phen)_3]^{2+}$ samples were performed in the absorption band of the metal complex (425 nm for the Λ and 470 nm for the Δ complex). As has been shown by Hiort et al.,[8] the complexes represent local binding geometries on DNA that are essentially independent of the drug/DNA ratio. It is therefore justified to use the LD in the absorption region of the bound metal complex as a measure of the total DNA orientation. This procedure was necessary in the gel experiments owing to the extensive ultraviolet absorption by the metal complex obviating LD measurements in the DNA base band. The rise and decay times of the electric field were less than 1 ms when they were measured over the cell electrodes. The LD signals were recorded on a Nicolet 2090 oscilloscope. Each measurement was repeated four times to improve the signal to noise ratio and the average LD response was used in the data evaluation. Presented data are in turn averages of measurements in at least two different gels.

2.5. Data Analysis. The electrophoretic migration in agarose gel of a long DNA like T2 DNA leads to a motion where the DNA conformation changes in a cyclic manner between extended and compact conformations, a mode of motion called reptation.[21−32] The average period time, $\langle T \rangle$, of the cycles has been found to be equal to the time t_u to reach the orientation undershoot in the oscillatory LD response, which is observed when a constant field is applied to a sample of the molecules in the electrophoresis gel.[32,33] It will be shown below that the LD responses from the T2 DNA complexed with $[Ru(phen)_3]^{2+}$ also exhibits the characteristic oscillations of cyclic migration. In our analysis we will therefore assume that

$$\langle T \rangle = t_u \tag{4}$$

also holds for the complexes.

By defining L as the migrated distance (the step length) during the period time T, the average step length $\langle L \rangle$ can be calculated from t_u and the measured steady-state mobility μ as[34]

$$\langle L \rangle = \mu E t_u \tag{5}$$

where E is the field strength in the experiment and μ is the electrophoretic velocity divided by the field strength. For native DNA an increase in field strength reduces $\langle T \rangle$ and increases $\langle L \rangle$, whereas an increase in DNA size increases both.[34]

3. Results

3.1. Flow Linear Dichroism. Figure 1 shows flow linear dichroism spectra of T2 DNA in the presence of the two enantiomers of $Ru(phen)_3^{2+}$ compared with spectra of uncomplexed DNA. The results confirm the conclusion by Hiort et al.[8] that the Δ, but not the Λ, enantiomer perturbs the DNA orientation: at a drug/DNA-phosphate ratio of 0.05 which is relevant for our electrophoretic studies, the DNA signal between 220 and 300 nm is reduced by approximately 50% for the complex with Δ (Figure 1a), while it is practically unaffected with Λ (Figure 1b), compared to the spectrum of free DNA. In

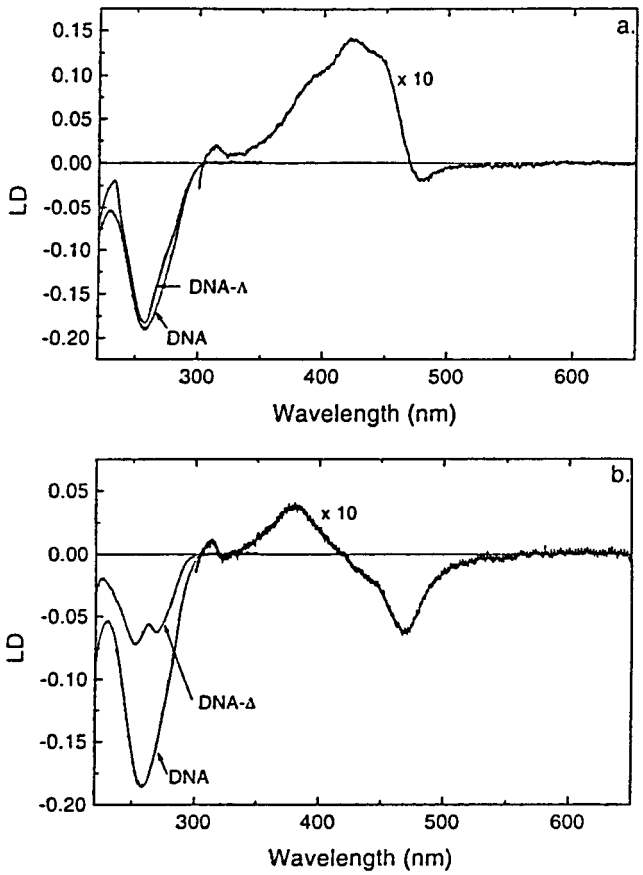

Figure 1. Flow linear dichroism (LD) spectra of pure T2 DNA and T2 DNA in the presence of (a) Λ-$[Ru(phen)_3]^{2+}$ and (b) Δ-$[Ru(phen)_3]^{2+}$. DNA concentration in bases: 100 μM. Metal complex/nucleotide binding ratio: 0.05. Optical path length: 0.1 cm. Shear gradient: 300 s^{-1}.

the 400 nm region are seen LD signals from the metal complex itself (revealing that it is bound and oriented by the DNA). The Δ complex is characterized by a positive LD peak at 380 nm and a negative peak at 470 nm, and the Λ complex by a large positive peak at 425 nm and a small negative band at 480 nm. The LD signals at 470 and 425 nm, respectively, were used in the electrophoresis experiments for the assessment of the DNA orientation since the 260 nm band of DNA was hard to measure in the presence of absorption from the metal complex.

3.2. Electrophoretic Mobility. Figure 2a shows the mobility of DNA, free as well as complexed with Δ- or Λ-$[Ru(phen)_3]^{2+}$, as a function of DNA length and at a field strength of 7 V/cm. For the pure DNA the mobility decreases monotonically with DNA length in the region below 23 kbp, above which limit it is practically constant. In presence of the Λ enantiomer the DNA mobility displays a very similar length dependence but is overall reduced some 10% compared with pure DNA. The Δ complex shows the same mobility as Λ for the 4, 6, and 9 kbp DNA, whereas for larger DNA its mobility is gradually decreased. The difference in mobility between Δ and Λ for the 164 kbp T2 DNA amounts to 20%. Figure 2b shows the data from Figure 2a normalized to the mobility of the shortest 4367 bp fragment. For comparison, results have been included from experiments where $[Ru(phen)_3]^{2+}$ was replaced by the structural analogue $[Ru(phen)_2DPPZ]^{2+}$ (DPPZ = dipyridophenazine), which is known to bind to DNA by classical intercalation.[35,36] The experimental conditions in these runs were the same as with $[Ru(phen)_3]^{2+}$ except that the concentration of $[Ru(phen)_2DPPZ]^{2+}$ in the gel was kept lower (3 μM) due to its higher affinity for DNA. Both the Δ and Λ enantiomer of $[Ru(phen)_2DPPZ]^{2+}$

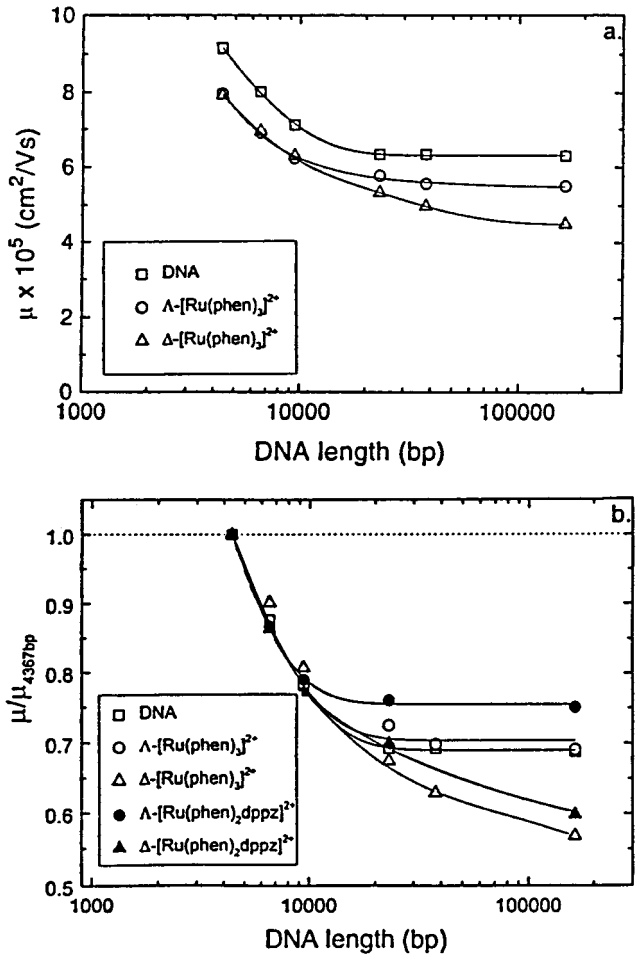

Figure 2. (a) Constant field mobility versus DNA size for pure DNA (□) and DNA in the presence of Δ-$[Ru(phen)_3]^{2+}$ (△) and Λ-$[Ru(phen)_3]^{2+}$ (○), respectively. DNA sizes: Hind III fragments of λ DNA (4.36, 6.59, 9.42, and 23.1 kbp), T7 DNA (37.9 kbp), and T2 DNA (164 kbp). Agarose concentration: 1%. $[Ru(phen)_3]^{2+}$ concentration: 9 μM. Electric field strength: 7 V/cm. (b) Mobility data from (a) normalized to the mobility of the shortest 4367 bp fragment. Results are also included from measurements performed under the same experimental conditions as in (a) but with the DNAs in the presence of Δ-$[Ru(phen)_2DPPZ]^{2+}$ (filled △) or Λ-$[Ru(phen)_2DPPZ]^{2+}$ (filled ○). $[Ru(phen)_2DPPZ]^{2+}$ concentration: 3 μM.

reduces the mobility of all DNA sizes (not shown). For the shortest DNA the reduction is largest with Λ (about 40% compared to about 30% with Δ) but for the longest DNA the reduction is also in this case significantly larger with the Δ enantiomer (about 40% compared to about 35%). Furthermore, as can be seen from the figure, the shape of the mobility curve for the respective complex is very similar to that of the corresponding complex with $[Ru(phen)_3]^{2+}$.

Figure 3 shows how the mobility of T2 DNA, with and without $[Ru(phen)_3]^{2+}$, varies with the field strength in the range 5−17 V/cm. Both the Δ and the Λ enantiomer reduces the mobility. However, the field-strength dependence of the mobilites of free DNA and as complex with Λ are similar, whereas the DNA in the presence of Δ deviates by displaying a reduction in mobility that is stronger at low fields than at high fields. As a consequence the difference in mobilities between the complexes has a maximum at low fields and vanishes at about 20 V/cm, where the curves meet each other.

3.3. Electrophoretic Orientation. Figure 4 shows the buildup of DNA orientation after a constant field has been applied to a zone of native or modified T2 DNA (164 kbp). The nonmonotonic rise profile of the LD, showing an overshoot and an undershoot before reaching a steady-state orientation, is typical for large DNA.[21−25,37] The over- and undershoot reflect the stretching and coiling, respectively, of the reptating DNA chains, and although they are seen only in the initial part of the LD response, their presence is evidence of ongoing cyclic migration.[37] The orientational response of the DNA−$[Ru(phen)_3]^{2+}$ complexes is very similar to that for pure DNA, which shows that the basic cyclic mode of migration is not altered by the bound metal complexes.

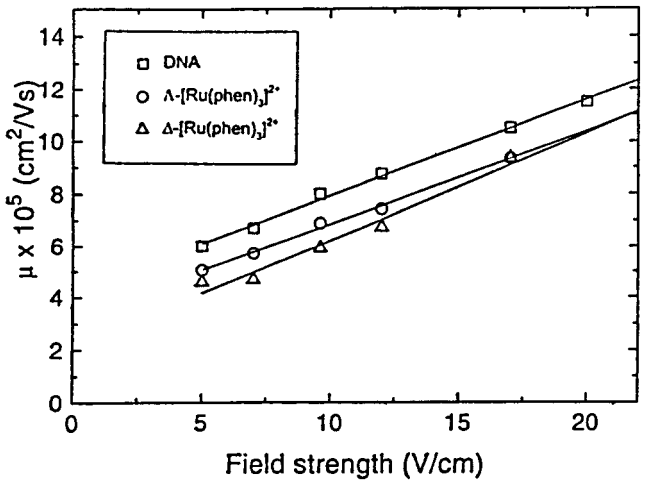

Figure 3. Constant field mobility versus electric field strength for pure T2 DNA (□) and T2 DNA in the presence of Δ-$Ru(phen)_3]^{2+}$ (△) and Λ-$Ru(phen)_3]^{2+}$ (○) respectively. Agarose concentration: 1%. [Ru-$(phen)_3]^{2+}$ concentration: 9 μM.

The time to the undershoot, t_u, was evaluated from orientation responses such as those in Figure 4 in order to obtain data on the average period time $\langle T \rangle$ (eq 4). Figure 5a shows that for both DNA-Λ and DNA-Δ the period time decreases with increasing field, as is the case with free DNA. This behavior reflects that on the average the migration cycle is completed in a shorter time if the field strength is raised; i.e., the stronger field pulls the molecules faster through the scenario of conformational changes. The detailed effects of the two metal complexes are more clearly seen in a plot of t_u relative to its value for free DNA (Figure 5b). Both Λ and Δ always make the cycle last longer. Λ lengthens the cycle to nearly the same degree (about 50%) at all fields, whereas Δ lengthens it more at high fields (nearly 65%) than at low fields (55%).

Figure 6 presents the values of the step length $\langle L \rangle$ calculated from eq 5 by use of data from Figures 3 and 5a. $\langle L \rangle$ increases with increasing field for both native and modified DNA. For the native form the step length increases from about 30 μm at 5 V/cm to about 37 μm at 17 V/cm, values in good agreement with earlier results.[13] DNA-Λ displays a field dependence similar to that of native DNA but the step lengths are about 30% longer. For DNA-Δ the steps are also throughout longer than for native DNA, but the field dependence is quite different. DNA-Δ starts with a step length that is shorter than for DNA-Λ and only about 10% longer than for the native DNA. With increasing field $\langle L \rangle$ increases to a greater extent than for DNA-Λ, the curves cross each other at about 12 V/cm and at the highest field $\langle L \rangle$ is almost 10% longer than for DNA-Λ and 50% longer than for native DNA.

For native DNA the relaxation of the orientation after the field is turned off occurs via one fast step, which is believed to relieve the stretching of the DNA along the path of the tube, and one very slow step, which has been shown to be well

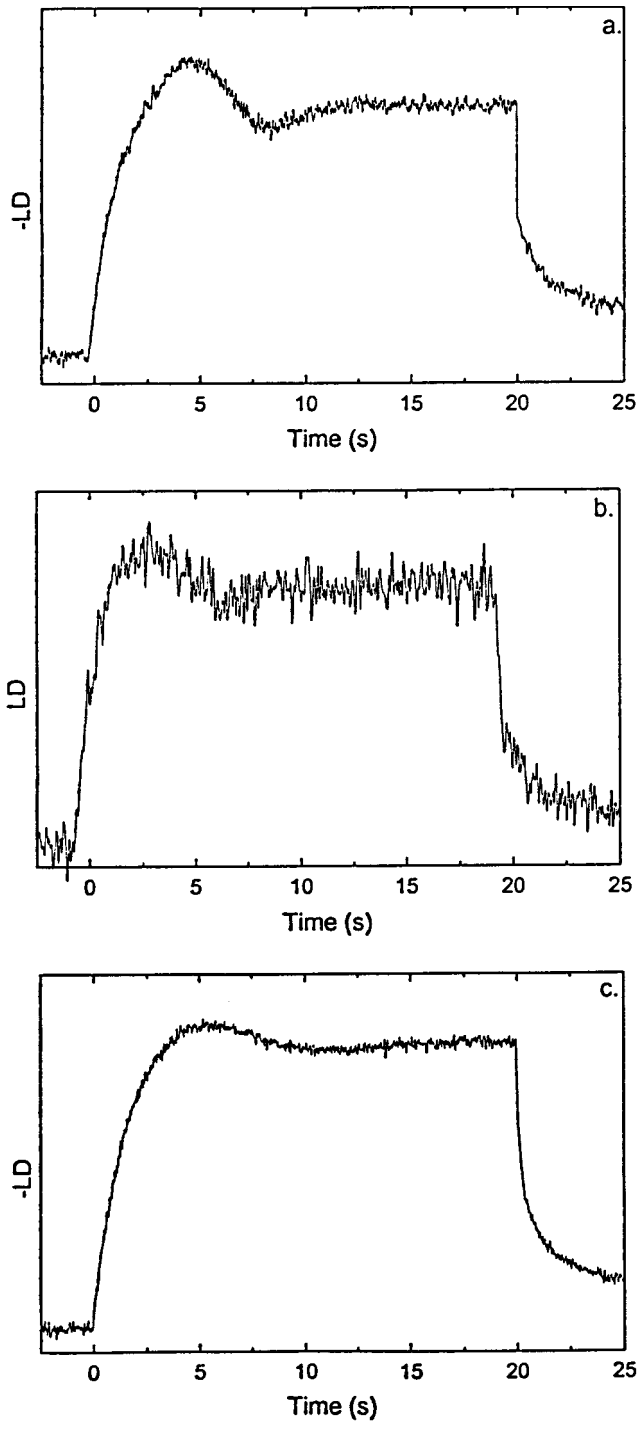

Figure 4. Typical LD responses of (a) pure T2 DNA, (b) T2 DNA complexed with Λ-$Ru(phen)_3]^{2+}$, and (c) T2 DNA complexed with Δ-$Ru(phen)_3]^2$ in 1% agarose gel when an electric field pulse (10 V/cm) is applied. The LD was measured at 260, 425, and 470 nm, respectively. The field free time before the pulses were applied was 10 min. $[Ru(phen)_3]^{2+}$ concentration: 9 μM.

described by reptation of the chain into an unoriented tube.[25,26] Also in the presence of $Ru(phen)_3{}^{2+}$ the DNAs exhibit biphasic LD relaxation, indicating the same type of relaxation behavior. We have studied the reptation component by a technique developed earlier.[22] If a second pulse of the same polarity and field strength is applied shortly after the first pulse is turned off, the over- and undershoot (peak-to-trough value = ΔLD) in the LD response are much less pronounced than in the response to the first field pulse if this is applied to the DNA system in its equilibrium state in the gel. With increasing waiting

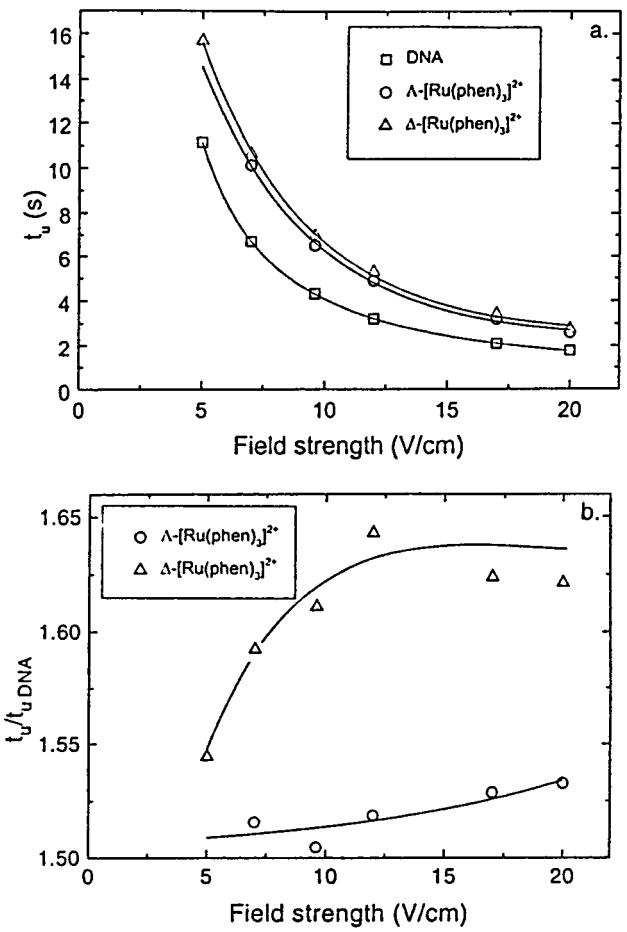

Figure 5. Field strength dependence in (a) t_u and (b) the ratio t_u for DNA in the presence of $Ru(phen)_3]^{2+}$ to t_u for native DNA. Native T2 DNA (□) and T2 DNA in the presence of Δ- (△), and Λ-$Ru(phen)_3]^{2+}$ (○), respectively. Agarose concentration: 1%. $[Ru(phen)_3]^{2+}$ concentration: 9 μM.

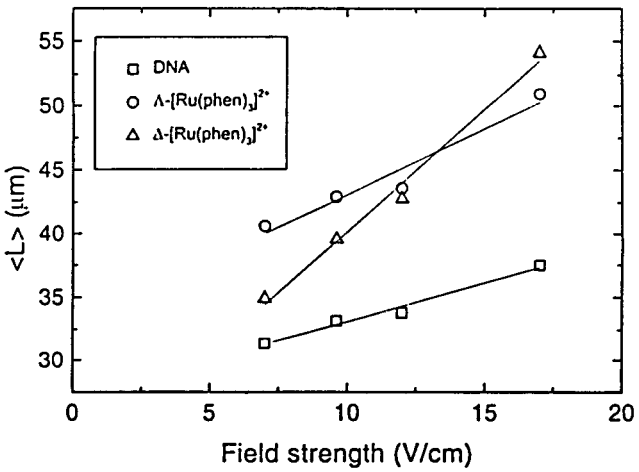

Figure 6. Field strength dependence in average step length ⟨L⟩. Native T2 DNA (□) and T2 DNA in the presence of Δ-$[Ru(phen)_3]^{2+}$ (△) and Λ-$[Ru(phen)_3]^{2+}$ (○), respectively. Agarose concentration: 1%. $[Ru(phen)_3]^{2+}$ concentration: 9 μM. The step length is calculated from eq 5, section 2.5, by use of data from Figures 3 and 5a.

time ΔLD increases until finally the value (ΔLD_{max}) corresponding to the equilibrium state of the DNA system is recovered. Figure 7 shows plots of $-\ln(1 - \Delta LD/\Delta LD_{max})$ versus waiting times for native and modified DNA, and it can be seen that the ΔLD recovery for all DNAs can be satisfactorily described by a single-exponential process. From slopes of the

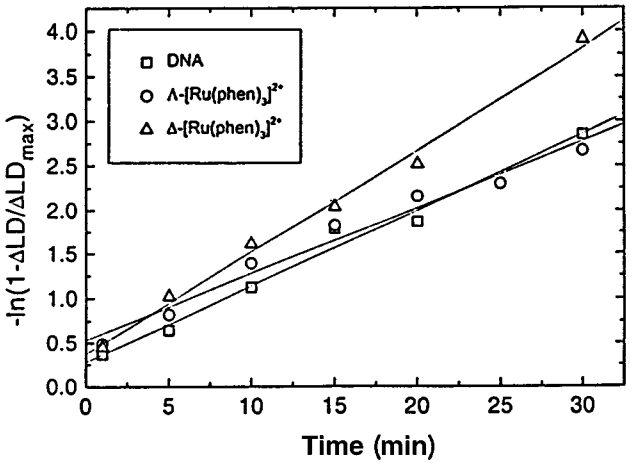

Figure 7. Semilogarithmic plot of the ΔLD recovery in 1% agarose gel for pure T2 DNA (□) and T2 DNA in the presence of Δ-[Ru(phen)$_3$]$^{2+}$ (△) and Λ-[Ru(phen)$_3$]$^{2+}$ (○), respectively. The slopes correspond to $\tau_{rec} = 11.2$ min for pure DNA and to $\tau_{rec} = 9.0$ and 13.2 min for DNA in the presence of Δ- and Λ-[Ru(phen)$_3$]$^{2+}$, respectively. [Ru(phen)$_3$]$^{2+}$ concentration: 9 μM.

plots the relaxation times, τ_{rec}, for Λ and Δ modified DNA were calculated to 13.5 and 8.7 min, respectively, and for uncomplexed DNA to 11.6 min, where the latter is in good agreement with earlier results.[13,22]

4. Discussion

The electrophoretic behavior in gels of a flexible polyelectrolyte like DNA is related to the electrokinetic charge, the contour length, and the flexibility of the molecule. Binding of a drug to the molecule may affect some or all of these properties and to a degree that will depend on the amount of bound drug. For Ru(phen)$_3^{2+}$ the binding constants to DNA at the ionic strength used in our experiments (about 25 mM), and the neighbor exclusion numbers, have been reported to be 4.9×10^4 and 2.8×10^4 M^{-1}, and 3.7 and 3.4, for the Δ and Λ isomers, respectively.[38] Calculations based on these values, and the value of the free concentration of the enantiomers in our experiments (9 μM), show that the number of bound molecules per 100 DNA phosphate groups has been around 4 in the DNA-Δ and around 3 in the DNA-Λ complexes. This, and the fact that the two enantiomers have the same formal charge (+2), means that the degree of reduction in the total charge of DNA is about 8% and 6%, respectively. However, the electrokinetic charge is in general not identical with the stoichiometric charge, and a change in the latter one need not necessarily give the same change in the former one. Not all counterions are site bound to the DNA, and part of the electric force on the mobile counterions reaches DNA through friction and electrostatic interactions. These effects, which influence the mobility, are sensitive not only to the number but also to the nature and distribution of both fixed charges and bound counterions. However, as is discussed below (section 4.1) our mobility results show that the degree of reduction of the electrokinetic charge of DNA is very equal in the two complexes. Furthermore, a scanning force microscopy study by Coury et al.[2] has shown that neither the binding of Δ nor of Λ leads to any apparent change of the contour length of the DNA chain. The differences we observe between the electrophoretic behaviors of the complexes should therefore be due mainly to differences in the effect of Δ and Λ on the flexibility of the DNA chain.

4.1. Mobility. At the field strength (7 V/cm) and gel concentration (1%) used in the mobility experiment shown in Figure 2a, the uncomplexed DNAs shorter than about 3 kbp migrate as isotropic coils, those between about 3 and 7 kbp as anisotropically deformed coils, and the longer ones as reptating chains where the reptation is most pronounced for the fragments longer than about 23 kbp.[37] These differences in the mode of migration are reflected in the shape of the mobility curve. The decrease in mobility with increasing size of DNA is strongest in the migration regime where DNA forms isotropic and anisotropic coils, less strong when DNA begins to reptate, and the mobility becomes essentially independent of size in the regime where the reptating mode of migration is pronounced. As seen in the figure the binding of Λ-Ru(phen)$_3^{2+}$ to DNA reduces the mobility of all DNA sizes but the shape of the mobility curve is very similar to that of the uncomplexed DNA. This similarity indicates that the Λ form does not change the mode of migration of neither short nor long DNA. For the longest DNA in the study (T2 DNA, 164 kbp) this is also confirmed by the LD measurements (section 3.3). The Δ form reduces the mobility of the shorter DNAs to the same extent as Λ but for the longer DNAs (which reptate in their native forms) there is a further, gradual reduction and for T2 DNA the mobility difference between the Δ and Λ complexes amounts to no less than 20%. However, the similar behavior of the shorter DNAs and the fact that *T*2 DNA reptates also in this case (section 3.3) suggest that Δ, like Λ, does not change the mode of migration of any of the DNA sizes.

Δ and Λ decrease the mobility of the smaller, nonreptating DNAs to the same degree (Figure 2a). In our earlier study with Pt compounds bound to DNA[13] (performed under the same experimental conditions and with the same DNA sizes as here) it was found that changes in the flexibility of DNA had nearly no effect on the mobility of these DNAs. This, and the fact that neither Δ nor Λ changes significantly the contour length of DNA (see above), shows that the decrease in mobility must be an effect of the reduction Δ and Λ give rise to in the electrokinetic charge of DNA, a reduction that must be similar for the two enantiomers since they decrease the DNA mobility to the same degree.

The difference between Δ and Λ, i.e., the extra reduction in mobility of large reptating DNA in the presence of the Δ enantiomer, must be an effect of a difference in flexibility of the DNA complexes since the charge reduction is similar in the two complexes and neither Δ nor Λ changes the contour length of DNA. The general reduction in mobility, as observed with the Λ complex, can be ascribed to the reduction of the effective charge of DNA. In our earlier study[13] it was found that in the reptation regime kinks gave a gradual reduction in the mobility of larger DNA very similar to that obtained here for the Δ complex, a similarity which indicates that Δ kinks the DNA. Flexible bends also reduced the mobility of larger DNA but to a much less extent than kinks, and the flexible joints had no apparent effect. The finding that the enantiomeric difference is mimicked by the classically intercalating [Ru(phen)$_2$DPPZ]$^{2+}$ complex (Figure 2b) suggests that the explanation for the kinks concluded for Δ is not simply a wedging semi-intercalation but most probably involves the diastereomeric interaction of the outer phenathroline ligands with the groove walls.

Also the field-strength dependence of mobility of T2 DNA (Figure 3) is similar for free DNA and for DNA complexed with Λ, whereas the DNA in the presence of Δ displays a mobility that increases more strongly with the field than free DNA. In our earlier study[13] the introduction of flexible joints had no effect on the field dependence of the mobility of T2

DNA, flexible bends gave a mobility that increased less, and kinks gave a mobility that increased stronger with the field, than for the native DNA. The behavior of the Δ complex is thus also in this respect similar to that shown by kinked DNA.

In summary, the DNA size dependence of the mobility, and the field dependence of the mobility of a long reptating DNA clearly suggest that the binding of Δ kinks the DNA, a conclusion that finds support also in our orientational measurements (see below). For DNA complexed with Λ these dependencies are very similar to those shown by native DNA, indicating that Λ does not change the flexibility of the DNA. However, in some cases, mobility alone cannot detect perturbations in the DNA helix[13] and, as will be shown below, also Λ affects the flexibility of DNA.

4.2. Period Time, t_u, and Step Length, $\langle L \rangle$. Both Λ and Δ increase the period time, t_u, of the cyclic conversion of the reptating T2 DNA (Figure 5). For DNA-Δ the field strength dependence in the ratio t_u/t_{uDNA} is similar to that shown by kinked DNA,[13] which further supports our conclusion above that Δ kinks DNA. The reduction in the effective charge of DNA due to the binding of the enantiomers is expected to increase t_u, as is observed, since it decreases the electric force, and thereby the velocity, with which the electric field drives the molecule through the reptation cycle. Furthermore, a reduction in charge is equivalent to a reduction in electric field strength, which increases t_u for native DNA (Figure 5a). However, a reduced field also reduces the step length of native DNA, whereas the binding of both Λ and Δ lengthens the step (Figure 6). An increase of the contour length of DNA, which increases both the period time and the step length,[34] could be an explanation to the longer steps but conflicts with the results of Coury et al. that neither Δ nor Λ changes the length of the DNA chain.[2] However, for native DNA there are strong indications that the step length is mainly determined by how far down the gel the arms of the molecules reach in the approximately U-shaped molecules that are formed when the molecules become hooked on gel fibers.[34] This in turn is determined by the length of the reptation tube that can be significantly shorter than the contour length of the DNA. The average contour length of the DNA chain per tube segment (the length of the gel pore) depends on the chain flexibility and the degree of stretching of the chain along the reptation tube. It is reasonable to assume that this is valid also for the DNAs with the bound enantiomers.

As shown above, the mobility data suggest that Δ induces kinks in the DNA. This will reduce the tube length of the relaxed chain since it increases the average contour length of the DNA chain per tube segment. If the resistance toward stretching is not affected, the reduction in tube length should therefore lead to a shortening of the step length, not a lengthening, as is observed here. However, the fact that the step length for DNA-Δ increases faster with increasing field than for free DNA (Figure 6) suggests that Δ makes it easier to stretch the DNA. The longer steps may therefore very well be consistent with a reduction of the effective length of the relaxed DNA since the DNA-Δ complex may be more stretched than native DNA during the migration. That the forces available for stretching DNA during electrophoresis in agarose gel reach levels, also at low fields, that can overcome length-reducing effects of flexibility changes was demonstrated in our study on DNA with bound Pt compounds.[13] With kinks (and also with flexible bends and flexible joints) the step length of the molecule was found to increase faster with increasing field than for native DNA, as is observed here for DNA-Δ. Furthermore, the step length became longer than that for native DNA at a field strength of about 10 V/cm although the kinks reduced the tube length of the relaxed chain by as much as 40%. In the present study DNA-Δ takes steps that already at the lowest field in our measurements (7 V/cm) are longer than the steps taken by native DNA. However, kinks induced by different compounds may, for example, have different bending angles and different rigidity, properties that can be expected to affect the stretching during electrophoresis. There are also indications that the reduction in the effective length of DNA caused by Δ is rather small at the relatively low binding ratio in our experiments (see section 4.3 below).

For the DNA-Λ complex the steps are also longer than for native DNA (Figure 6). However, the increase in the step length with increasing field strength is in this case more similar to that for the native DNA, which shows that the two molecules have a similar stretching behavior within the studied field strength interval. If this is so also at lower fields, the longer steps cannot be due to a difference in stretching between the two molecules but is most likely due to a difference in the tube length of their relaxed chains; the tube must in that case be longer for the complex than for the native DNA. This points to (since the contour length of the complex and of free DNA should be very similar[2]) Λ making the DNA molecule less flexible; i.e., it stiffens the chain (increases the tube length by decreasing the average contour length per tube segment), a conclusion that finds support in our overshoot recovery experiments (see section 4.3 below). The fact that the stretching behavior of the DNA is nearly the same as for the native form indicates that the stiffening of the chain is locally limited to the binding sites of the metal complex and therefore, due to the low affinity of the complex to the DNA, leaves the main part of the DNA chain unaffected.

The binding of both Δ and Λ increases strongly the period time, t_u, of the DNA (Figure 5). If the reduction of the effective charge of DNA should be the sole reason for the increase, this should have the same magnitude in the two cases since the enantiomers reduce the charge to the same degree (section 4.1). However, Δ increases t_u more than Λ, and the field dependence in t_u is also different for the two DNA complexes. This shows that not only is there the reduction in the charge that affects t_u but also the changes in helix flexibility caused by the enantiomers must have an effect. The separate contributions from the two effects to the increase of t_u have been estimated as follows. The mobility of the small, nonreptating DNAs in the experiment shown in Figure 2 is reduced by 10% by both Δ and Λ. This reduction is due mainly to the decrease in the effective charge of DNA (see section 4.1 above). To obtain, under the same experimental conditions (7 V/cm, 1% agarose), a similar reduction in mobility of these DNAs by a change in the electric field strength, this has to be decreased by about 15% according to mobility data presented by Hervet and Bean.[39] A corresponding reduction in the field strength on the long reptating T2 DNA gives (at the same experimental conditions) a 30% increase in t_u, which shall be compared with the more than 50% increase given by the enantiomers (Figure 5). That it takes longer times for DNA-Δ and DNA-Λ than for native DNA to complete the migration cycle is thus to a large part due to the influence of Δ and Λ on the helix flexibility. The kinks induced by Δ probably increase t_u by increasing the friction between the DNA and the gel fibers.[13] Enhanced friction may provide the extra resisting force, and the slow-down of the sliding of the U-shaped molecules, so that the field can, and has time, to stretch the modified DNA more efficiently than the native DNA. In the

DNA-Λ case, on the other hand, the stiffening of the helix caused by Λ must have a small effect on the sliding friction since the stretching behavior of this molecule is similar to that of native DNA. This indicates that the increase in t_u due to the stiffening of the chain is mainly a result of the increase this causes in the effective length of the chain.

4.3. Reptation Time. For native DNA the relaxation time, τ_{rec}, for the overshoot recovery (see section 3.4) agrees well with the time, τ_R, it takes for the chain to reptate into an unoriented tube after the field has been turned off,[22] and we will assume here that this also holds for the modified T2 DNAs. τ_R is given by [22]

$$\tau_R = \frac{L_c^3 b\zeta}{a^2\pi^2 kT}$$

where L_c is the contour length of the chain, b is the length of segments (b is equal to two persistence lengths), a is the mesh size of the gel, and ζ is the translational friction coefficient for a segment. Since neither the binding of Δ nor of Λ leads to any apparent change of the length of the DNA chain,[2] we will assume that L_c has the same value for the two modified DNAs as for the native DNA. The stretching behavior of DNA-Δ during the electrophoresis indicates, as was discussed above, that the kinks induced by Δ enhances the friction between the chain and the gel fibers. However, this does not necessarily mean that they also enhance it in absence of the electric field. During electrophoresis the electric field presses the chain against the gel fibers and the contact between the kinks in the moving chain and the fibers should be of a much longer duration than in absence of field because the Brownian motion then more easily can move the interacting kinks away from the gel fibers. The presence of kinks should therefore not have any large effect on the friction of the DNA chain during diffusion. The stretching behavior of DNA-Λ indicates, as was also discussed above, that the stiffening of the DNA chain caused by Λ has a small effect on the friction of DNA during electrophoresis, and there are no reasons to believe that the effect is larger during diffusion. We will therefore assume here that the friction f per length unit ($f = \zeta/b$) has the same values for the DNA complexes as for the native DNA. With $\tau_R = \tau_{rec}$, this leads to the conclusion that the ratio between the b values (and thus the persistence lengths) for the modified and the native DNA is equal to the square root of the corresponding ratio between their relaxation times τ_{rec}. Insertion of the measured τ_{rec} values (section 3.3) gives that the Δ decreases, and Λ increases the persistence length by in both cases about 10% (equivalent with a reduction and an increase, respectively, of about 7% of the tube length of the relaxed chain[40]). The values should be taken as estimates due to the approximations in the calculations. They support, however, our conclusions above that Δ kinks DNA and that Λ stiffens the DNA helix.

5. Concluding Remarks

The results presented above, together with those obtained in our earlier study using Pt compounds as ligands, demonstrate that the effect of ligands on helix flexibility can be studied by measuring their effect on electrophoretic mobility and orientational dynamics of reptating DNA in gel and employing a simple model for the relation between mobility and orientational dynamics in the analysis of the measurements. The method gives so far only qualitative information due to lack of quantitative models for the effects of helix extension and helix flexibility. To arrive at such models more experiments and systematic studies are needed not only on DNA complexed with ligands with known effects on DNA structure but also on native DNA.

The results further indicate that the effect of Δ-$[Ru(phen)_3]^{2+}$ on the hydrodynamic properties of DNA is due to a kinking but that this kinking rather appears to be effected by the outer, nonstacked phenanthrolines than to a wedging semi-intercalation of the inner phenanthroline.

In the study where the Pt compounds were used these were covalently bound to the DNA chain. The ligands used in this study, the Δ and Λ enantiomers of $[Ru(phen)_3]^{2+}$, bind reversibly and with low affinity to DNA. Such ligands will dissociate from DNA during a normal gel electrophoresis run since their residence occupancy times on DNA are much shorter than the electrophoresis time. The present study clearly demonstrates, however, that also reversible DNA−ligand complexes can be analyzed, both regarding mobility and orientational dynamics, in the same way as a covalent complex provided the ligand also is present in free form in the electrophoresis solution and precautions are taken to keep its concentration along the gel constant during the electrophoresis.

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