Nitroxide-Labeled Ru(II)-Polypyridyl Complexes as EPR Probes To Study Organized Systems. 2. Combined Photophysical and EPR Investigations of B-DNA

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Abstract: We report here the application of two polypyridyl complexes of ruthenium(II), $Ru(phen)_2(phen\text{-}T)Cl_2$ and $Ru(bpy)_2(phen\text{-}T)Cl_2$, to the examination of the interactions of the family of polypyridyl metal complexes with B-DNA. Phen-T is a modified 1,10-phenanthroline ligand where T is a stable nitroxide (TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl) which is covalently attached to the phenanthroline unit via a carbamate linkage. These nitroxide-substituted ruthenium complexes are unique in that the same compound is a probe which can be monitored by two completely independent spectroscopic techniques. We report here the comparison of time-resolved luminescence measurements and electron paramagnetic resonance (EPR) spectra in the presence of B-DNA and confirm that the data obtained using both methods are mutually consistent. The EPR spectra provide independent evidence for two distinct modes of binding of these complexes with DNA: one surface and the other intercalative. The EPR spectra have been evaluated to determine the rotational correlation times of motion of the bound radicals; all experimentally recorded EPR spectra have been successfully simulated.

Introduction

The photophysical behavior of polypyridyl complexes of ruthenium(II) and their interactions with microheterogeneous structures have been extensively studied in the last decade.[1] The ^{3}MLCT (metal-to-ligand charge transfer) states of the ruthenium complexes are characterized by a broad absorption in the 430-480 nm range and an emission in the 590-700 nm range. The ^{3}MLCT transitions of Ru(II) complexes, and the corresponding lifetimes, are strongly dependent on the local environment due to the influence of the environment on nonradiative vibrational deactivation pathways.[1] This deactivation pathway is inhibited by the process of interaction at a negatively charged surface, such as intercalation in DNA, and leads to an increase of the ^{3}MLCT excited-state lifetimes. The characteristic behavior observed during the interaction of $Ru(phen)_3^{2+}$ (phen = 1,10-phenanthroline) and B-DNA,[2] SiO_2,[3] micelles,[4] polymer and polyelectrolyte solutions,[5] or starburst dendrimers with carboxylate head groups[6] is the enhancement of steady-state luminescence (compared to these in "free" solution) and biexponential lifetime decays when more than one binding mode is possible.

The accepted paradigm for the interaction of Ru(II)-polypyridyl complexes with anionic charged microstructures such as DNA, micelles, starburst dendrimers, or polyelectrolytes suggests that the probe may be located in at least three conceptually distinct regions:[7] (1) the probe may be unbound and "free" in the bulk solution; (2) the positively charged Ru(II) probe may be associated with the negatively charged interface by electrostatic binding (this may be either territorial binding in the Gouy-Chapman layer where diffusional motion is relatively unrestricted or it may be ionic site binding next to the surface); and (3) the Ru(II) probe may be directly but noncovalently bound to the microstructure. Scheme I presents a more detailed pictorial representation of this division into different binding sites.

At least two major modes of binding by polypyridyl complexes of ruthenium(II) to B-DNA have been described.[2] One of these is surface binding, comparable to the binding seen with other negatively charged microstructures. The second mode of binding is a more rigid form of interaction that involves the intercalation of one of the flat aromatic phenanthroline units between two DNA base pairs. These forms of binding involve not only electrostatic attractions between the oppositely charged DNA and ruthenium complexes but also hydrophobic and dipole-dipole interactions.

These features of the binding of Ru(II) complexes with DNA have been convincingly revealed from photophysical experiments. $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) shows no evidence for strong binding to DNA; however, upon binding to DNA, a distinct change in the photophysical properties of $Ru(phen)_3^{2+}$ and other Ru(II) complexes has been noted.[2] There is a hypochromic shift in the UV/vis band corresponding to the ^{3}MLCT state; the steady-state luminescence intensity increases by a factor of almost 2, and the luminescence decay of the photoexcited state changes from a clean monoexponential process to a trace which can be modeled by a biexponential decay process. Whereas the components with the shorter lifetimes, which are very similar to these found in homogeneous solution (τ_s = 400-600 ns), have been assigned to a

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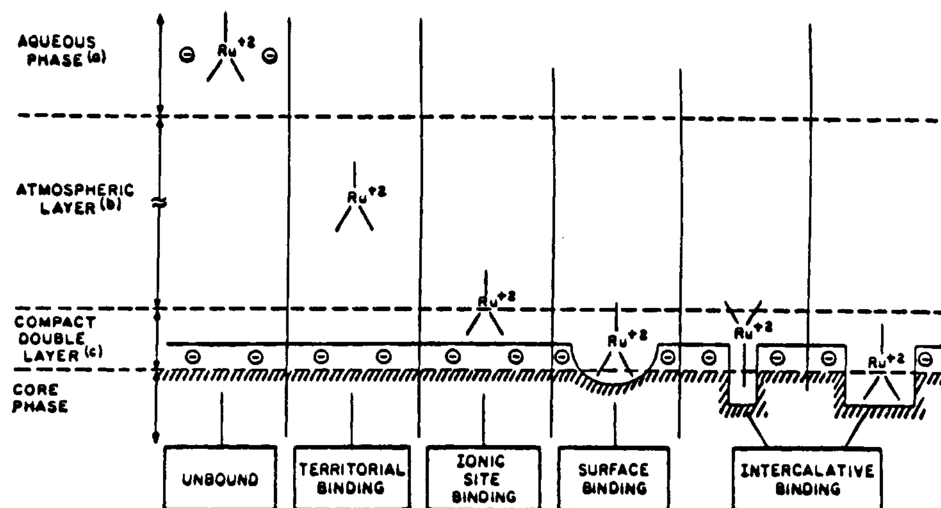
Scheme I



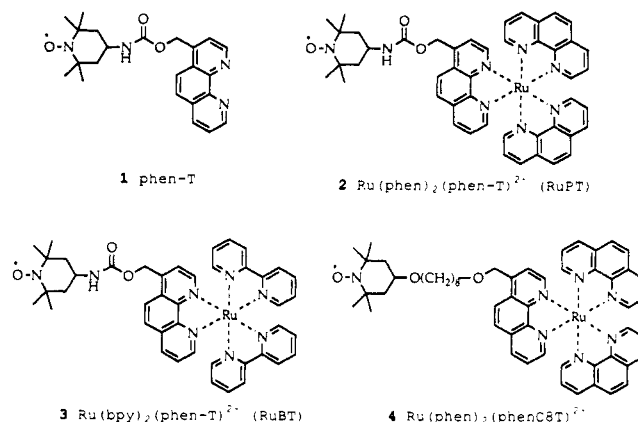
surface-bound species, the longer lived component, which shows a lifetime enhancement (τ_1 = 1600–2300 ns), has been assigned to an intercalatively bound species.[2] Both components are differentially quenched, as compared to free $Ru(phen)_3^{2+}$, by anionic quenchers, and only the longer lived component retains polarization in the emitted light when excited with polarized light.

An intrinsic drawback of all experiments that rely on the presence of a molecular probing device to evaluate characteristic properties of microenvironments is the extent of the perturbation of the system itself in the presence of the molecular probe. Therefore, results obtained using external probes must necessarily be treated with circumspection, and supportive evidence from different techniques and measurements is both germane and prudent. These perturbations are more important if the probing device is similar in size to the microstructure with which it is interacting. For instance the size of $Ru(phen)_3^{2+}$ (diameter ~18 Å) is comparable with the size of the smallest sodium alkyl sulfate micelles ($C_7H_{15}OSO_3Na$ or $C_8H_{17}OSO_3Na$) as well as the "early" generations of starburst dendrimers.[6]

For B-DNA, which is a large macromolecule, the "overall perturbation" of the system using $Ru(phen)_3^{2+}$ as a probing device may be considered to be small, but the local changes during the intercalation of $Ru(phen)_3^{2+}$ can be significant.[2] For instance, intercalation leads to a partial unwinding of the double helix and therefore may modify the local motion of the base pairs near the site of intercalation.[8]

Electron paramagnetic resonance (EPR) spectroscopy using stable nitroxides as spin probes[9] was chosen as a second method of probing the binding of Ru complexes to DNA which was independent from any form of "optical" spectroscopy. Not only does the nitroxide EPR probe report on the polarity of the surrounding medium (reflected by changes in the hyperfine coupling constant of the electron spin with the nuclear spin of nitrogen atom), but the diffusional restriction and motional anisotropy experienced by the probe determine the shape of the three-line pattern.[9]

The novel complexes $Ru(phen)_2(phen\text{-}T)^{2+}$ (**2**) and $Ru(bpy)_2(phen\text{-}T)^{2+}$ (**3**) have already been used for probing alkyl sulfate micelles and the micellization process in the presence of the Ru complexes by EPR.[10] The results were in very good agreement with the earlier findings from photophysical experiments.[6c,11] These new ruthenium complexes were obtained by synthesizing a 1,10-phenanthroline derivative that had a nitroxide moiety covalently attached to it via a carbamate linking unit; we refer to this ligand as phen-T (**1**). Starting from this ligand the complexes $Ru(phen)_2(phen\text{-}T)^{2+}$ (**2**) and $Ru(bpy)_2(phen\text{-}T)^{2+}$ (**3**) were obtained using standard procedures. A slightly different ligand was used to make a $Ru(phen)_3^{2+}$ derivative where the nitroxide was separated from the complex by a linker containing eight methylene units, $Ru(phen)_2(phenC8T)^{2+}$ (**4**).



1 phen-T

2 $Ru(phen)_2(phen\text{-}T)^{2+}$ (RuPT)

3 $Ru(bpy)_2(phen\text{-}T)^{2+}$ (RuBT)

4 $Ru(phen)_2(phenC8T)^{2+}$

It is well-known from the literature that TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) is able to quench the photoexited states of ruthenium complexes.[12] However, we see no evidence of intramolecular quenching in our complexes, and their photophysical behavior is similar to the underivatized complexes which are lacking a TEMPO moiety. This fact allows us to conduct both photophysical and EPR measurements on the same system, allowing for two completely different routes by which to glean information.

The vast majority of EPR investigations of nucleic acids to date have involved spin-labeling rather than the spin probe technique, and several reviews of this literature exist.[13] The work of Bobst and co-workers[14] exemplifies this approach; it involves the synthesis of nitroxide-labeled bases and then incorporation of the monomers

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into DNA polymers enzymatically. Careful control of stoichiometry is required to prevent incorporation of large amounts of label, which could result in exchange effects. These DNA segments have been investigated by EPR to monitor base dynamics,[14a] local Z-DNA conformations,[14b] and B- to Z-DNA transitions.[14c] Short oligomers containing a spin-labeled deoxyuridine moiety have also been prepared and used as an EPR probe of DNA hybridization.[14d]

An approach which lends itself to more control over the specific site of the spin label has been developed by Hopkins and co-workers: they have synthesized a nucleoside in which a nitroxide is attached to a thymidine base through an acetylenic linker.[15a-c] This nucleoside can be converted to a phosphoramidite which may be used in solid-phase synthesis. Using this compound they have prepared and studied several oligomers and have shown that the EPR spectra for single strands, double strands, and double strands where the spin probe lies in a loop are all characteristically different.[15b,c]

Lerman and co-workers have looked at the EPR of dye intercalators modified with a nitroxide spin label; these include acridine,[16a] ethidium,[16b] and propidium[16a] derivatives. They have examined the EPR spectra of DNA-intercalator complexes in solutions and in fibers and have concluded that the correlation times experienced by the probe are associated with torsional movements in the helix and are not dependent on the free motion of the probe. The use of spin probes, which are randomly distributed, allows for an estimation of both the partitioning of the probe in different environments and of the different abilities to interact at the different sites.

The interaction of our TEMPO-attached complexes with DNA provides a unique system to study the behavior of small-molecule binding agents with double-helical DNA using two independent methods. Such investigations permit an independent assessment of binding modes using different techniques which may weigh the contributions of each binding mode to different extents. Lastly, the well-established coordination chemistry of these systems makes a systematic study both synthetically feasible and accessible.

Analysis of the EPR Spectra

Two main parameters can be evaluated from the EPR spectra: (1) the isotropic hyperfine coupling constant, variations in which can be correlated to variations in the polarity of the environment felt by the nitroxide unit, and (2) the correlation times for the reorientation motion (τ), which are expected to increase upon binding of the Ru-TEMPO assembly to DNA and therefore provide direct information about the binding strengths in the different binding modes.

The simple methods of analyzing correlation times of motion based upon the dependence of the line width on the nitrogen quantum number[17] are not applicable in these studies since all of the EPR spectra recorded in the presence of DNA were obviously the superposition of at least two different components, one of which was in a faster motion condition than the other.

For a more accurate analysis of the line shape, the spectra have been fully simulated with the program developed by Schneider and Freed.[18] The key parameters that were checked to improve the fitting between the experimental and calculated spectra were the **g** and **A** tensor components (evaluated from computing the

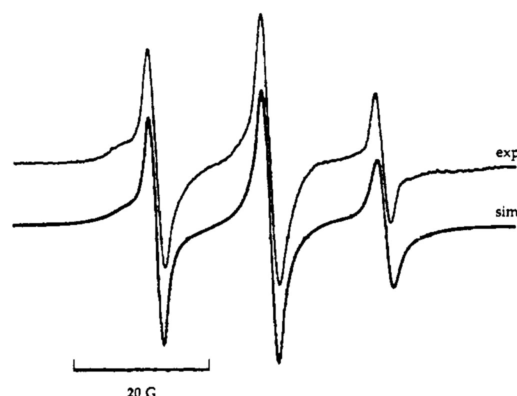

Figure 1. Experimental (top curve) and simulated (bottom curve, sum of three components) EPR spectra of $Ru(phen)_2(phen\text{-}T)^{2+}$ (50 μM) in the presence of DNA (1 mM phosphate) in Tris buffer (NaCl, 50 mM). See text for details of computation.

spectra in the slowest motion), the model assumed for the rotational diffusional motion (Brownian, Free, or Jump), the principle components of the diffusion tensor (including the diffusion tilt angle with respect to the magnetic frame and taking due consideration of both the mean rate of diffusion and the anisotropy of motion), parameters related to an ordering potential, the intrinsic line width, and the Heisenberg spin exchange frequency.

In the cases where we obtained more than one signal contributing to the overall EPR spectrum (arising from $Ru(Phen)_2(phen\text{-}T)^{2+}$ bound in different modes to DNA), each signal was computed separately and then added in a correct ratio to reproduce the experimentally recorded spectra. This complication is usually not seen with the spin-labeling technique, where all of the probes appear to experience an average environment.[14b,c,15] Also, a subtraction–addition procedure was carried out on the different components of the spectra as a further check on the reliability of the computation.

Experimental Section

All EPR spectra were recorded on a Bruker ESP300 instrument interfaced to a computer with the ESP1600 software system. Temperature control was achieved with the Bruker ER 4111T temperature controller ($T = 20 \pm 1$ °C).

The synthesis of the ligand phen-T (**1**) and the complexes RuPT and RuBT will be described elsewhere. The concentrations of the complexes were estimated spectrophotometrically; for $Ru(bpy)_2(phen\text{-}T)^{2+}$ (RuBT) it was assumed that its extinction coefficient at 452 nm was the same as that of $Ru(bpy)_2phen^{2+}$ (ϵ = 16 000 M^{-1} cm^{-1}), and for RuPT and **4** it was assumed that their extinction coefficients at 443 nm were the same as that of $Ru(phen)_3^{2+}$ (ϵ = 20 000 M^{-1} cm^{-1}).[2c] Calf thymus DNA was obtained from Pharmacia-LKB and extensively dialyzed against Tris buffer (50 mM NaCl, 5 mM Tris, pH = 7.0) prior to use, and concentrations were estimated spectrophotometrically (ϵ_{260} = 6600 M^{-1} cm^{-1}).[19] $K_3Cr(CN)_6$ was synthesized according to reported procedures.[20]

The concentrations used in a typical EPR experiment were as follows: DNA (phosphate), 1 mM; Ru complex, 50 μM; 5 mM Trizma/HCl buffer (pH = 7); NaCl, 50 mM or the described concentration of salt added. The concentrations used in a typical photophysical experiment were as follows: DNA (phosphate), 200 μM; Ru complex, 10 μM; 5 mM Trizma/HCl buffer (pH = 7); NaCl 50 mM or the described concentration of salt added. All solutions were prepared in deionized water (Millipore). The ratio of [DNA]/[Ru] was held constant at 20 for all experiments.

Time-resolved lifetime measurements were conducted using a single photon counting (SPC) unit consisting of an Edinborough 199F nanosecond flash lamp, Ortec electronics, and a Tractor-Northern TN-1710 multichannel analyzer interfaced to an IBM PS/2 Model 70 computer. The decay traces were analyzed using a computer program generously provided by Professor F. C. DeSchryver of the University of Leuven, Belgium.

Results and Discussion

A sequence of "typical" DNA-probing experiments were carried out with $Ru(phen)_2(phen\text{-}T)^{2+}$ and $Ru(bpy)_2(phen\text{-}T)^{2+}$ using the

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EPR and SPC techniques. Figure 1 presents, as the top curve, the EPR spectrum of 50 μM $Ru(phen)_2(phen-T)^{2+}$ in the presence of 1 mM calf thymus DNA in a solution of 5 mM Tris buffer (pH = 7) and 50 mM NaCl. These conditions of buffer, salt, and [DNA]/[Ru] = 20 are the standard conditions under which we have performed most of our photophysical experiments reported in earlier studies.[2] It is clear upon inspection of Figure 1 that *at least* two components exist that contribute to the observed EPR spectrum; one of the components is in a slower motion condition relative to the other. This complication of more than one component is usually not considered in the spin-label studies due to the relative "homogeneity" of the sample.

The bottom curve in Figure 1 shows a simulated spectrum that attempts to reproduce the experimentally recorded spectrum of the top curve in Figure 1. Although the excited-state decay in emission by this probe in the presence of DNA can be satisfactorily fit with a biexponential decay function, all attempts to generate the simulated EPR spectrum by the addition of only two components did not lead to satisfactory reproductions of the experimental curve. Only the addition of three components led to reasonable reproductions of experimental spectra as judged by a visual comparison of the superposition of the two spectra; no least-squares analyses were performed. Further support of this finding is provided by the subtraction procedure: the spectrum obtained by subtracting the experimental spectrum obtained under conditions when binding is severely inhibited (at high NaCl concentrations, vide supra) from the spectra at lower salt concentrations clearly shows the presence of two components and facilitates their computation. It is noteworthy that three components have also been seen in the case of spin-labeled ethidium bound to DNA, even though the binding constant of ethidium to DNA ($K = 3 \times 10^6$ M^{-1})[21] is almost 3 orders of magnitude larger than that for $Ru(phen)_3^{2+}$ ($K = 3300$ M^{-1}).[2c] Experiments with sulfate micelles[10] have shown that the addition of the TEMPO unit tends to make RuPT slightly more hydrophilic relative to $Ru(phen)_3^{2+}$. Therefore, it might be expected that the binding of RuPT to DNA is diminished relative to $Ru(phen)_3^{2+}$; however, evidence from photophysical experiments (vide supra) suggests that the perturbation in binding behavior is very small at best.

The values of the **g** tensor and hyperfine tensor that were used for calculating all three components are the following: $g_{xx} = 2.0094$, $A_{xx} = 6.8$ G; $g_{yy} = 2.0064$, $A_{yy} = 8.2$ G; $g_{zz} = 2.0028$, $A_{zz} = 36.7$ G. These values were used earlier for the simulation of spectra of a nitroxide interacting with micellar systems.[22] The use of the same **g** and A tensors to calculate all contributions to the observed spectra is an approximation that is not critical since our aim is to delineate between the *relative* mobilities and distributions of the probes at the different sites. Indeed the same line-to-line splitting in the experimental spectra, which also allows for the subtraction of one spectrum from another, is supportive of the invariance of the A tensor, which in turn is a probe of the environmental polarity.

The experimental spectra are characterized by narrow lines, and all of the components were computed using an intrinsic line width of 1.0 G. Despite the use of low relative concentrations of the probe ([DNA]/[Ru] = 20), it is possible that aggregation of the complexes at the DNA surface could occur. This concern was unfounded since the sharpness of the lines requires that no significant spin–spin dipolar broadening be present in these systems, ensuring that the radicals bound to DNA are not present at distances short enough that spin–spin interactions may be manifested.

Brownian motion was used in computing all three spectra, as has been done in computing the spectra of the ethidium spin sprobe in the presence of DNA.[16b] All three components have characteristic features, and we shall refer to them as components A, B, and C. Component A is distinguished by fast ($\langle\tau\rangle = 150$ ps, $\tau_\parallel = 75$ ps, and $\tau_\perp = 300$ ps) and low anisotropic motion conditions, i.e., $N = 4$ where N is the ratio of the parallel to the perpendicular components of the diffusional motion tensor; component B displays slower motion ($\langle\tau\rangle = 2.25$ ns, $\tau_\parallel = 1.12$ ns, and $\tau_\perp = 4.5$ ns) which still exhibits low anisotropy ($N = 4$), while component C displays slow motion ($\langle\tau\rangle = 3$ ns, $\tau_\parallel = 474$ ps, and $\tau_\perp = 19$ ns) which is highly anisotropic ($N = 40$). Components A and B have the z axis as their main axis of rotation and are relatively insensitive to the anisotropy of motion. Component C on the other hand has the y axis as its main axis of rotation. The relative contributions of these three components in the curve presented in Figure 1 are 72:13:15.

The EPR method, we believe, is providing us with a sensitive probe of the different modes of binding. The $Ru(phen)_3^{2+}$ complexes which are surface bound cannot be photophysically distinguished from those that are free in solution, except in the presence of quenchers. However, here we may ascribe component A of the EPR spectra to those complexes in the bulk solvent, while component B may arise from the surface-bound component (Scheme I). The binding of the species, resulting in component B, is evidenced by an almost 10-fold drop in its correlation time for motion relative to component A; however, the lack of motional anisotropy suggests that the binding does not impose great restraints on the rotational orientation of the probe molecule. Component C may be attributed to the intercalated form of the molecule, and this is reflected in the large value for the motional anisotropy ($N = 40$). This value of $N = 40$ implies that the perpendicular component of the rotational diffusional tensor is close to rigid motion conditions ($\tau = 19$ ns). *This is the first report of direct evidence, to the best of our knowledge, where Ru complexes which are free in solution, surface bound to the DNA, and intercalatively bound to the DNA have been experimentally distinguished.*

Spin labels on short oligomers (12- and 24-mers) have been reported to have rotational correlation times of 4–7 ns.[15b,c] Although these times are similar to what we observe, the values in the literature have been ascribed to the overall motion of the DNA moving as a rotating cylinder, on the basis of estimations from hydrodynamic calculations. For natural DNA, which is several hundred base pairs long, the numbers for the rotation of the DNA are much longer (>200 ns).[16a] Hence it is unlikely that the rotational correlation times we are recording are due to the motion of the entire DNA as one rotating unit. The rotational correlation time for isotropic motion that was calculated for the ethidium spin probe in a disodium ethylenediaminetetraacetic acid buffer, when bound to DNA from chicken erythrocytes, was $\tau_R = 30$ ns.[15b] Considering all of the differences in the substrate, probe (especially the thousand-fold greater binding affinity of ethidium over $Ru(phen)_3^{2+}$), experimental conditions, and methods of estimations, we believe these two times to be in reasonable agreement for the same motion. This correlation time has been attributed to a torsional motion of the base pairs relative to one another and hence is reflected in the intercalated probe.[16]

The effect of the restricted motion that is experienced by the nitroxide is due to the carbamate linkage around which there is hindered rotation because of the amide bond. This is supported by the fact that the EPR spectrum of the complex $Ru(phen)_2(phenC8T)^{2+}$, where the nitroxide is separated from the phenanthroline by eight methylene units, in the presence of DNA is essentially identical with that seen in solution. This may be rationalized by the fact that the linker is long and flexible enough so that, even though the complex is bound to the DNA, the nitroxide unit does not feel any influence of that binding. This approach is similar to the "dip-sticking" strategy devised by Bobst and co-workers, who found that the mobility of the spin label sharply increases once the length of the connecting tether is long enough that the nitroxide may access a region outside the major groove of the DNA helix.[23a] A similar dependence of the motion of the nitroxide on the length of the tether connecting it to the

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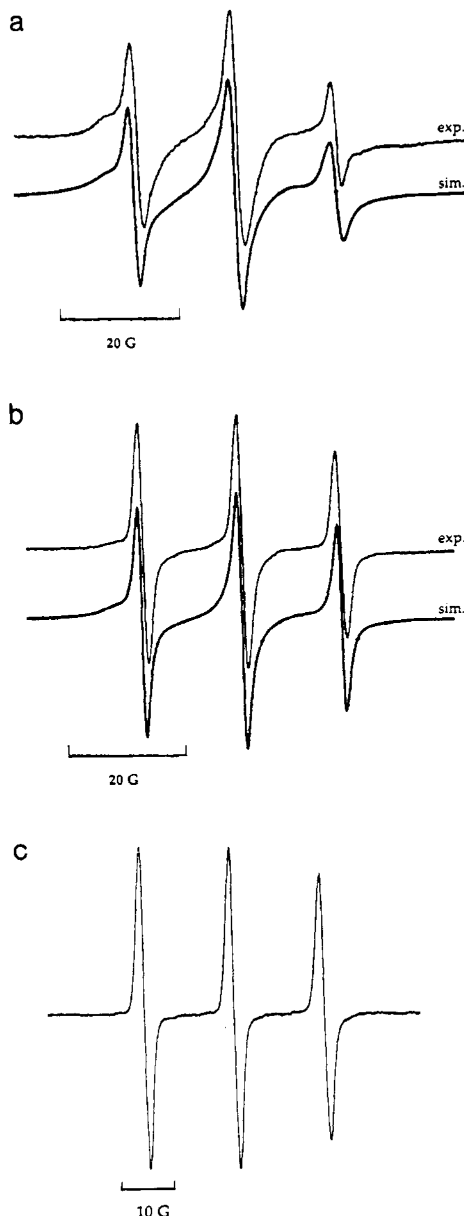

Figure 2. Experimental and simulated EPR spectra of Ru(phen)$_2$-(phen-T)$^{2+}$ (50 μM) in the presence of DNA (1 mM phosphate) in Tris buffer: (a) 10 mM NaCl; (b) 100 mM NaCl; (c) 50 mM Mg^{2+}.

oligonucleotide has also been noted by Hopkins and co-workers with their spin labels.[23b] The dependence of the EPR spectrum on the length of the tether is also important in establishing the fact that the motional restriction experienced by **2** is due to some motion coupled with the DNA and not an independent motion of the probe, which should be tether length independent.

Salt Effect on the Binding of RuPT to DNA

EPR Experiments. The ionic strength of the surrounding electrolyte is known to have a profound influence on the interactions of charged species with one another.[24] A higher ionic strength is known to reduce the repulsions between similarly charged species while it reduces the effective attraction between oppositely charged species. Furthermore, the exact nature of the ions making up the electrolyte is germane too since it has been documented that 1 equiv of a divalent ion has a greater influence than 2 equiv of a monovalent ion.[24] Schulte-Frohlinde and coworkers[2f] have investigated such effects in the interaction of Ru–polypyridyl complexes with DNA; they report a diminution in the binding of the complexes to DNA as the concentration of the salt in the buffer increases. Also, salts with divalent cations such as Mg^{2+} and Ca^{2+} are far more effective at disturbing the binding of these probes to DNA than is Na^+.

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Table I. Calculated Rotational Correlation Times and Relative Percentages of the Different Contributions of Ru(phen)$_2$(phen-T)$^{2+}$ on DNA at Different Salt Concentrations

	$\langle\tau\rangle$, ns[a,b]			% component		
NaCl, mM	A	B	C	A	B	C
10	0.15	2.25	3.0	64	17	19
50	0.15	2.25	3.0	72	13	15
100	0.04	2.0	3.0	85	3	12
200	0.03	2.0	3.0	90	2	8

[a] The values reported in table are mean rotational correlation times $\langle\tau\rangle = (\tau_\parallel\tau_\perp)^{1/2}$. [b] The main axis of rotation for components A and B is the z axis, while that for component C is the y axis.

Table II. Lifetimes and Percentage Contributions of Short (τ_s) and Long (τ_l) Lived Components of Ru(phen)$_2$(phen-T)$^{2+}$ on DNA at Various Salt Concentrations

NaCl, mM	τ_s, ns	% τ_s	τ_l, ns	% τ_l
0	549	27	1496	73
50	535	41	1530	59
100	515	56	1477	44
200	503	73	1208	27

To confirm our assignment of the EPR components A, B, and C to the free, surface-bound, and intercalatively bound species, respectively, we decided to carry out a similar investigation on the effect of salt on the binding of our probe to DNA. Figure 2a,b presents the experimentally recorded and calculated spectra of RuPT on DNA at 10 and 100 mM NaCl. Inspection of these spectra reveals that there is an almost complete disappearance of the broad underlying slow motion component in the 100 mM NaCl spectrum relative to the 10 mM NaCl spectrum. Also, comparison of the 10 mM NaCl spectrum with the spectrum shown in Figure 1 recorded at 50 mM NaCl reveals, by visual inspection, that the relative contribution of the slow motion component is larger when the concentration of the salt is lower. Figure 2c is the spectrum recorded under conditions of 50 mM Mg^{2+} where the slow motion component is not in evidence.

Table I presents the relative contributions of components A, B, and C recorded for salt concentrations of 10, 50, 100, and 200 mM NaCl. Also presented are the correlation times of motion that were used for each of these components in the different salt conditions. The data in Table I are supportive of our initial assignent presented above. As the salt concentration increases, the contribution of component A monotonically increases while that of components B and C decreases. The surface-bound component should be more sensitive, relative to the intercalated component, to the salt effects: this is seen by a more rapid decrease in the contribution of component B to the overall spectrum relative to component C. Similar results have been seen in the investigation of the salt effect of the ethidium spin probe.[16b] The contribution of the bound ethidium is seen to decrease over a hundred-fold on going from 0.2 to 200 mM NaCl.

A more subtle feature lies in the observation that the value of $\tau_\perp$ of component A drops from 300 to 60 ps on going from 10 to 200 mM NaCl. Thus the EPR measurements reveal that the unbound component of the Ru complexes is not really "free" but is probably in the atmospheric collar of ions that is loosely associated with the polyanionic DNA. That the corresponding correlation times for components B and C are essentially invariant with the salt conditions is consistent with there being a stronger binding of the species, responsible for components B and C in the presence of DNA.

Photophysical Experiments. Table II presents the values of the lifetimes and the relative contributions of the short- (τ_s) and long-lived (τ_l) components of RuPT when bound to DNA at the same salt concentrations at which the EPR measurements were recorded. Qualitatively, one sees a trend that is analogous to the results from the EPR experiments: the contribution of the

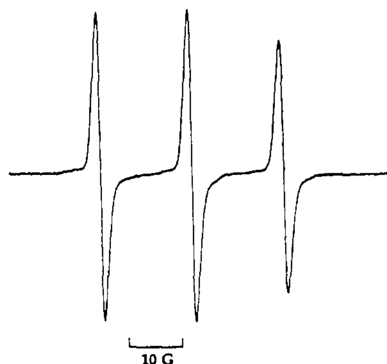

Figure 3. Experimental EPR spectrum of $Ru(bpy)_2(phen\text{-}T)^{2+}$ (50 μM) in the presence of DNA (1 mM phosphate) in Tris buffer (NaCl, 50 mM).

long-lived component, which is ascribed to the intercalated form, decreases from 73% at 10 mM NaCl to 27% at 200 mM NaCl. That the nitroxide unit does not significantly perturb the binding abilities of the molecule can be ascertained by a comparison of the photophysical results for $Ru(phen)_3^{2+}$ and RuPT: the relative contributions of the long-lived components are 66 and 59%,[2a] respectively.

The photophysical experiments were performed with [Ru] = 10 μM instead of 50 μM as used for the EPR measurements to maintain a suitable optical density of the solutions at the wavelength of excitation ($A_{440} < 0.2$). All other parameters, however, were the same as in the EPR experiments. The lack of quantitative agreement of the photophysical and EPR results is not surprising since these are two completely unique techniques, each with its own characteristic time scales and responses. The qualitative parallelism, however, that can be drawn between these two techniques holds up remarkably well.

Influence of the Ligand on Binding Ability

Since the polarity of the surrounding medium is known to influence the hyperfine coupling of the spin with the nitrogen nucleus, we suggest that the nitroxide moiety is directed away from the DNA unit toward the aqueous phase because the same values of the hyperfine tensors can be used to simulate all three components. This would indicate that the phenanthroline ligand which bears the nitroxide unit is not involved in the intercalative mode of binding. More concrete evidence supporting this hypothesis was obtained by investigating the EPR behavior of $Ru(bpy)_2(phen\text{-}T)^{2+}$ in the presence of DNA.

Photochemical studies of $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$ in the presence of DNA show that $Ru(bpy)_3^{2+}$ does not undergo intercalative binding, as evidenced by the absence of a long-lived component in its luminescence decay[25] and the lack of any luminescence enhancement. This is understandable since the bipyridine ligand is known to be more hydrophilic and lacks the intercalative surface, present in the phenanthroline unit, for stacking interactions. We have also prepared the spin probe $Ru(bpy)_2(phen\text{-}T)^{2+}$ (refered to as RuBT), which is the EPR-active variant of $Ru(bpy)_2phen^{2+}$. Photophysical studies with $Ru(bpy)_2phen^{2+}$ reveal the presence of a long-lived component and luminescence enhancement, implying that the parent complex does undergo a small but significant extent of intercalation.[2b]

Figure 3 shows the EPR spectrum recorded for the complex RuBT in the presence of DNA. What is very clear is the absence *of any distinctive slow motion contribution to the spectrum.* This result is entirely consistent with our conclusion above that the nitroxide moiety prevents the ligand to which it is attached from participating in the intercalative binding mode. A luminescence enhancement of 1.18, as opposed to ~2 for intercalating complexes, has been recorded for RuBT, and a long-lived component with a very modest increase in its lifetime (τ_l = 900 ns; τ_l > 1800

(25) Although under low salt conditions a long-lived component has been reported[2f] for $Ru(bpy)_3^{2+}$, no such component has been detected by us under our conditions.

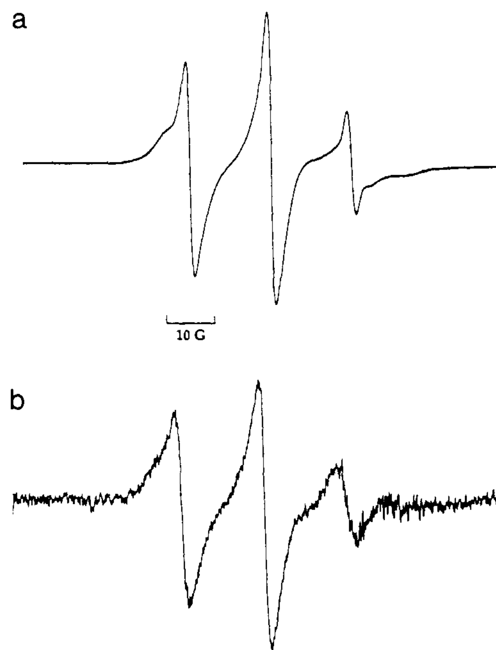

Figure 4. Experimental EPR spectrum of $Ru(phen)_2(phen\text{-}T)^{2+}$ (50 μM) in the presence of DNA (1 mM phosphate) in Tris buffer: (a) in the absence of $Cr(CN)_6^{3-}$; (b) in the presence of 10 mM $Cr(CN)_6^{3-}$ (after subtraction of the Cr signal).

ns for $Ru(phen)_3^{2+}$) is seen. Thus, although the parent compound ($Ru(bpy)_2phen^{2+}$) does exhibit intercalation into double-stranded DNA, the variant (RuBT) cannot. This result bolsters the conclusion from the photophysical experiments that it is the phenanthroline ligand, and not the bipyridine ligand, which is necessary for intercalative binding.

An EPR "Quenching" Experiment

One of the key experiments that helped in the assignment of the long-lived photophysical luminescence decay to a species that was strongly interacting with the DNA was a quenching study with charged quencher molecules. Since DNA is a polyanion, it effectively electrostatically shields any bound material from negatively charged species, while it enhances the influence of any positively charged species. The photophysical experiments involved the use of $Fe(CN)_6^{4-}$ as an anionic quencher and $Co(phen)_3^{3+}$ as a cationic quencher of excited-state $Ru(phen)_3^{2+}$. The result observed was that the emission polarization of a sample of $Ru(phen)_3^{2+}$ on DNA increased in the presence of $Fe(CN)_6^{4-}$.[2b] This is explained by the fact that the luminescence from the species in bulk solution, which should not be polarized, was quenched far more effectively than that of the species bound to the DNA, which should be polarized. On the other hand, when $Co(phen)_3^{3+}$ was used as the quencher, the emission polarization was seen to decrease[2b] since it was the DNA-bound Ru which was being quenched far more effectively than the Ru in the free solution.

We decided to mimic this photophysical experiment by performing a corresponding EPR "quenching" experiment. The EPR "quencher" would be a paramagnetic species which would effectively modify the relaxation rate of the electronic spin and hence the linewidth of the EPR signal; a negatively charged quencher should affect the relaxation of species which are furthest from the DNA, while a positively charged quencher should affect those species which are bound to DNA. We selected $Cr(CN)_6^{3-}$ as the anionic quencher. The broad signal arising due to the Cr complex is essentially the same both in the presence and absence of DNA. This allows us to subtract the spectrum of the Cr species recorded in water from the spectrum recorded for the DNA/Cr/RuPT mixture and obtain the pure "quenched" EPR spectrum of RuPT in the presence of DNA in the absence of the interfering Cr spectrum. The addition of 5 mM $Cr(CN)_6^{3-}$ to a solution of RuPT on DNA leads to an increase in the line width of component A from 1.0 to 2.0 G, *while the other two components remain*

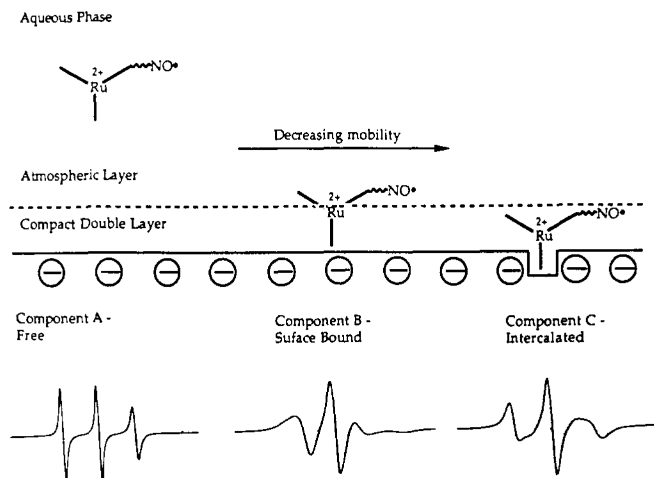

Figure 5. Schematic representation of the discernible binding modes of $Ru(phen)_2(phen\text{-}T)^{2+}$ on DNA and the corresponding calculated spectra for each component.

unaffected. Also, at this concentration of quencher the percentage contribution of component A decreases from 64 to 57%, while the relative ratios of components B and C are unaffected.

Figure 4a,b shows the spectra of RuPT on DNA recorded in the absence and presence (after subtraction of the Cr signal) of 10 mM $Cr(CN)_6^{3-}$, respectively. It can be seen that the signal in Figure 4b is dominated by that of the slow motion components and that the sharp line from component A has been significantly quenched out: the line width of component A increases to 2.5 G and its contribution in Figure 4b decreases to 40% from a value of 64% in Figure 4a. Although the relative contributions of components B and C are unaffected, the line width of component B now increases from 1 to 3.5 G by the addition of 10 mM Cr, while that of component C does not change. This result too is in agreement with our assignment that component B is less tightly bound and hence less well protected by the anionic DNA. It is not clear why the line width of component A is less than that of component B, but one possible reason could lie in the decreasing sensitivity of the calculations to the line width when the intensity of a particular component decreases.

Corresponding experiments, which attempted to quench selectively the slow motion components, using several cationic quenchers including $Ni(phen)_3^{2+}$ and Cu(II) provided results which were ambiguous at best. The reason for the ambiguity lies in the fact that the concentrations of the quenchers needed to produce any observable effect were too large, and it proved to be impossible to distinguish between EPR paramagnetic effects and those resulting from simple salt-induced displacements.

Conclusions

We have used novel ruthenium complexes which serve as both EPR and photophysical probes to monitor their interactions with double-helical B-DNA. The conclusions from the photophysical experiments presented here and earlier in the literature have been corroborated and further refined in the EPR investigations.

We have presented evidence that the polypyridyl complexes of ruthenium interact with DNA in two distinct modes which are different from "unbound" complexes. The bound modes are characterized by correlation times of motion which are at least 15 times slower than those seen in homogeneous solution. The two slow motion components are attributed to a surface-bound component, characterized by low motional anisotropy, and an intercalatively bound component, which is characterized by a large motional anisotropy. A third component which is attributed to "free" complex is also observed; however, we have presented evidence that even this component is not entirely independent of the DNA and is probably associated with the atmosphere of ions surrounding the DNA for salt concentrations less than 100 mM. The distinction between surface-bound and "free", which is not possible in the photophysical experiments, is clearly feasible in the EPR simulations. These conclusions are summarized in Figure 5, which presents the computed spectra for the individual components A, B, and C at 50 mM NaCl concentrations.

The rotational correlation times that are needed for the simulation of the slow motion components are comparable in value to those seen for spin labels which are covalently attached to the bases of oligonucleotide chains.[15] The isotropic rotational correlation time for the intercalated component may be equated with that of a spin-derivatized ethidium,[16b] and this has been correlated with a torsional motion of the base pairs relative to one another.

The short tether that links the nitroxide to the complex, with hindered rotation about the amide bond, is necessary to see the effects we observe, since a complex with a longer tether, **4**, made up of eight flexible methylene units, to the nitroxide does not display any evidence for slow motion components in the EPR spectra in the presence of DNA.

Consistent with the photophysical results is the observation of decreased binding of the complex in the presence of increasing salt concentrations; the sensitivity of the binding to the divalent cation Mg^{2+} is greater than that to the monovalent cation Na^+.

The intercalative binding mode does not involve the phenanthroline unit which has been modified with the nitroxide: this is supported by the fact that the spectra could be computed by assuming the same values of the hyperfine tensors for all three components. This indicates that the polarity experienced by the nitroxide unit in all three components is the same, and hence the radical in the intercalatively bound species should be directed out toward the solvent rather than into the DNA. Further, the complex $Ru(bpy)_2(phen\text{-}T)^{2+}$ shows no evidence for intercalative binding, despite the fact that the model compound $Ru(bpy)_2(phen)^{2+}$ does show photophysical evidence for intercalative binding. This is again consistent with the conclusion that the modification of the phenanthroline precludes RuBT from interacting strongly with the DNA.

We have carried out an EPR "quenching" experiment where $Cr(CN)_6^{3-}$, an anionic paramagnetic species, was used to selectively quench out the signals of the fast motion component A. This component is believed to arise from the species that is "free" in solution and hence is not protected, from the anionic relaxant, by the anionic phosphate groups on the DNA.

Thus, our spin/photophysical probes nicely serve the dual purpose of providing two independent means of probing the interactions of polypyridyl complexes of ruthenium with microheterogeneous media. The studies utilizing these complexes as spin probes serve as a nice complement to the elegant work involving the incorporation of nitroxides as spin labels to study nucleic acid structure. Our probes are more readily accessible and do not involve arduous synthetic efforts. Their ability to be probed by two independent techniques may be suitably exploited to clarify and reconfirm conclusions that are available through only one method. Further, they may be used to study any naturally occurring nucleic acid or protein and are not limited to only those substances which are amenable to being incorporated with a spin label. The true potential of these probes will be realized when their selectivity may be tuned to a fine degree, and it will be possible to target specific sites on the DNA duplex.

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