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Oxidative Damage by Ruthenium Complexes Containing the Dipyridophenazine Ligand or Its Derivatives: A Focus on Intercalation

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Interactions with DNA by a family of ruthenium(II) complexes bearing the dppz (dppz = dipyridophenazine) ligand or its derivatives have been examined. The complexes include $Ru(bpy)_2(dppx)^{2+}$ (dppx = 7,8-dimethyldipyridophenazine), $Ru(bpy)_2(dpq)^{2+}$ (dpq = dipyridoquinoxaline), and $Ru(bpy)_2(dpqC)^{2+}$ (dpqC = dipyrido-6,7,8,9-tetrahydrophenazine). Their ground and excited state oxidation/reduction potentials have been determined using cyclic voltammetry and fluorescence spectroscopy. An intercalative binding mode has been established on the basis of luminescence enhancements in the presence of DNA, excited state quenching, fluorescence polarization values, and enantioselectivity. Oxidative damage to DNA by these complexes using the flash/quench method has been examined. A direct correlation between the amount of guanine oxidation obtained via DNA charge transport and the strength of intercalative binding was observed. Oxidative damage to DNA through DNA-mediated charge transport was also compared directly for two DNA-tethered ruthenium complexes. One contains the dppz ligand that binds avidly by intercalation, and the other contains only bpy ligands, that, while bound covalently, can only associate with the base pairs through groove binding. Long range oxidative damage was observed only with the tethered, intercalating complex. These results, taken together, all support the importance of close association and intercalation for DNA-mediated charge transport. Electronic access to the DNA base pairs, provided by intercalation of the oxidant, is a prerequisite for efficient charge transport through the DNA π-stack.

Introduction

Charge transport through DNA has been shown to require proper stacking of the π-orbitals of the heterocyclic nucleobases.[1] When base bulges,[2] mismatches,[3,4] or nonaromatic residues[5,6] are inserted into the π-stack, charge transport is efficiently shut off. When properly stacked, the DNA π-array has been shown to mediate guanine oxidation at sites 200 Å from a remotely bound oxidant.[7,8] Oxidative damage to DNA from a distance is therefore necessarily sensitive to the intervening DNA sequence and structure.[9−12]

Besides proper π-stacking, energetic driving force is a requirement for charge-transfer reactions. Guanine is the easiest nucleobase to oxidize with a potential of 1.29 V versus NHE.[13] *Ab initio* molecular orbital calculations predict that in a 5′-GG-3′ guanine doublet, the bulk of the HOMO lies on the 5′-G, which has a lower oxidation potential than a single guanine.[14,15]

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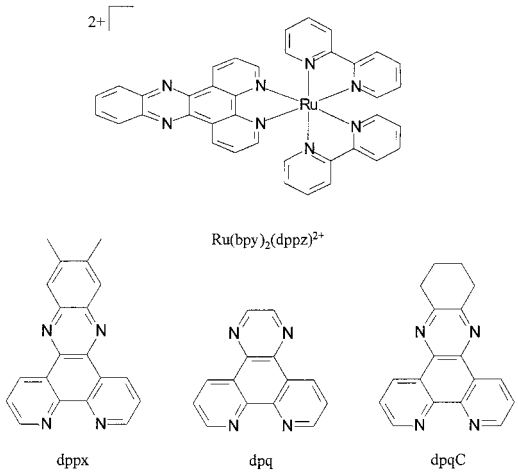
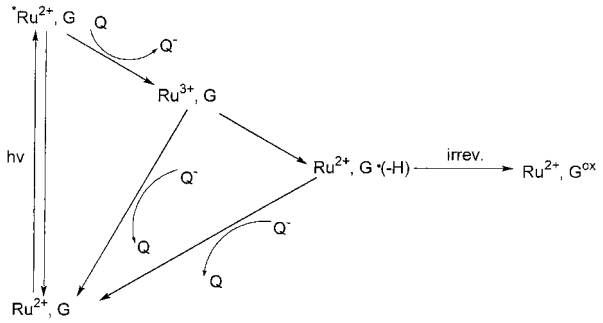


Figure 1. Schematic illustrations of metal complexes.

The work presented here was designed to address a third aspect considered to affect the oxidation of guanine sites in DNA: the ability of the oxidant to intercalate into the π-stack. This intercalation is proposed to result in more effective coupling to the DNA π-array and more efficient hole injection, facilitating long-range charge migration through the π-stack. To explore this hypothesis, octahedral ruthenium(II) complexes (Figure 1) bearing the intercalating dppz ligand or derivatives thereof, $Ru(bpy)_2(dppz)^{2+}$ (dppz = dipyridophenazine), $Ru(bpy)_2(dppx)^{2+}$ (dppx = 7,8-dimethyldipyridophenazine), $Ru(bpy)_2(dpq)^{2+}$ (dpq = dipyridoquinoxaline), and $Ru(bpy)_2(dpqC)^{2+}$ (dpqC = dipyrido-6,7,8,9-tetrahydrophenazine), have been prepared, and both their binding interactions and oxidative reactions with DNA have been characterized. Dppz complexes of ruthenium have been extensively studied owing to their unique luminescence properties when bound to DNA.[16,17] The binding of dpq and dpqC complexes of ruthenium to DNA has been explored structurally using NMR methods.[18,19] These complexes vary both in their ability to stack intercalatively within the DNA helix and in their efficiency in promoting oxidative DNA damage.

Oxidative damage to DNA is generated with these ruthenium complexes through a flash/quench experiment.[20] The flash/quench methodology, originally developed to explore charge transport reactions in proteins,[21] has been effectively applied in characterizing transient radical intermediates in the DNA charge transport process[6] and in generating protein/DNA cross-links.[22,23] Scheme 1 illustrates the series of reactions associated with the flash/quench

Scheme 1. Schematic Illustration of the Flash/Quench Methodology



experiment. The cycle is initiated by visible light, which excites the intercalated ruthenium(II) complex. The excited ruthenium(II) complex, *Ru(II), is then quenched by a nonintercalating electron accepting quencher, Q, such as $Ru(NH_3)_6^{3+}$, methyl viologen (MV^{2+}), or $Co(NH_3)_5Cl^{2+}$, so as to form Ru(III) *in situ*. This species can be reduced back to Ru(II) either through recombination with reduced quencher (Q^-) or by electron transfer with guanine (G). The oxidized guanine radical can then return to its resting state by reaction with reduced quencher or undergo further reaction to form a family of oxidative products, G_{ox}.[24]

In addition to Ru(II) complexes that contain derivatives of the dppz ligand, $Ru(phen)_3^{2+}$ and $Ru(bpy)_3^{2+}$, which possess the necessary driving force to oxidize DNA but do not intercalate as well as complexes containing the dppz ligand, have been examined. The importance of intercalation into the π-stack is also explored by comparing oxidative DNA damage resulting from covalently bound derivatives of ruthenium complexes containing bpy versus dppz ligands.

Experimental Section

Materials. $[Ru(bpy)_3]Cl_2$ and $[Ru(phen)_3]Cl_2$ were purchased from Aldrich and recrystallized from water prior to use. $[Ru(NH_3)_6]Cl_3$ was purchased from Aldrich and was used as received. Calf thymus DNA (ct-DNA) was purchased from Amersham and was dialyzed against a buffer of 20 mM sodium phosphate, 10 mM NaCl, pH 7.85 prior to use. Phosphoramidites were from Glen Research and were used as received.

Metal Complex Synthesis. The ligands dipyrido[3,2-a:2′,3′-c]-phenazine (dppz), 7,8-dimethyldipyrido[3,2-a:2′,3′-c]phenazine (dppx), dipyrido[3,2-*d*:2′,3′-*f*]quinoxaline (dpq), and dipyrido[3,2-*a*:2′,3′-*c*]-(6,7,8,9-tetrahydro)phenazine (dpqC) were prepared according to literature protocols,[18,19] as was $Ru(bpy)_2Cl_2 \cdot 2H_2O$.[25] The bis-(bpy) ruthenium complexes containing the third ligand L = dppz, dppx, dpq, or dpqC, $[Ru(bpy)_2(L)]Cl_2$, were synthesized by heating 1.2 equiv of L with $Ru(bpy)_2Cl_2 \cdot 2H_2O$ at 150 °C in ethylene glycol for 1 h. Solid NH_4PF_6 was used to precipitate the orange-red solid which was then washed with H_2O and diethyl ether. Complexes were purified on alumina columns equilibrated with dichloromethane and eluted with acetonitrile. Water soluble chloride salts

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were then obtained using a Sephadex QAE−25 ion-exchange resin equilibrated with 0.2 M KCl and eluted with acetonitrile. The three-ligand complex $[Ru(phen)(bpy')(dppz)]Cl_2$ (bpy′ = 4-butyric acid-4′-methyl-2,2′-bipyridine) was prepared according to literature procedures,[25,26] and $[Ru(bpy)_2(bpy')]Cl_2$ was prepared from $Ru(bpy)_2Cl_2 \cdot 2H_2O$.[25] For all complexes prepared, ^1H NMR and FAB-MS analyses agreed with values expected.

Extinction coefficients for complexes were obtained as follows: accurate measurements of ruthenium concentrations were made using a Perkin-Elmer/Sciex Elan 5000A ICP-MS and $[Ru(bpy)_3]Cl_2$ as calibrant, and absorbance measurements were collected using a Varian 300 Bio spectrophotometer. Ten replicates of concentration and absorbance for each sample were used to calculate extinction coefficients at 450 nm as follows: $Ru(bpy)_2(dppx)^{2+}$, $\epsilon = 21000 \pm 600\ M^{-1}\ cm^{-1}$; $Ru(bpy)_2(dpq)^{2+}$, $\epsilon = 14200 \pm 400\ M^{-1}\ cm^{-1}$; $Ru(bpy)_2(dpqC)^{2+}$, $\epsilon = 14300 \pm 500\ M^{-1}\ cm^{-1}$; $Ru(bpy)_2(dppz)^{2+}$, $\epsilon = 21400 \pm 600\ M^{-1}\ cm^{-1}$.

Electrochemistry. Ground-state oxidation and reduction potentials for the ruthenium complexes were obtained on a Bioanalytical Systems (BAS) model CV-50W electrochemical analyzer. A glassy carbon working electrode, Ag/AgCl reference electrode, and Pt auxiliary electrode were used in a single cell sample apparatus. Solutions of racemic metal complex (1 mM) in dry acetonitrile (Fluka; stored over molecular sieves) containing 100 mM tetrabutylammonium hexafluorophosphate were degassed with Ar prior to use, and voltammograms were collected using 100 mV/s scan rate. $E_{1/2}$ values were taken as the average of the voltage of maximum current for the forward and reverse electrochemical processes. Potentials are reported in volts versus NHE.

Luminescence. Emission and excitation spectra were obtained on an ISS-K2 spectrofluorometer. Emission intensities were determined by integration of the luminescence spectrum and standardized against $[Ru(bpy)_3]Cl_2$ as a calibration for the instrument. Solutions containing 10 μM racemic metal complex and 1 mM nucleotides ct-DNA in 20 mM sodium phosphate, 10 mM NaCl, pH 7.85 were excited at 450 nm, and emission was monitored from 500 to 800 nm. Excitation spectra were obtained by monitoring at the emission maximum while varying excitation wavelength from 250 to 600 nm. Luminescence polarization data were obtained using an ISS-K2 spectrofluorometer in an L-configuration. Samples consisted of 10 μM racemic metal complex in 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. When present, ct-DNA concentration was 1 mM nucleotides**,** and glycerol samples contained 60% glycerol by volume. Samples were irradiated at 450 nm, and emission was monitored at 610 nm using a 495 nm cutoff filter.

To determine excited-state lifetimes, time-resolved emission measurements were conducted using a pulsed YAG-OPO laser ($\lambda_{ex} = 470$ nm). Laser powers ranged from 3 to 4 mJ/pulse. To obtain luminescence lifetimes, τ, time-resolved emission data were fit to $y(t) = 100[C_1 \exp(-t/\tau_1) + (1 - C_1)\exp(-t/\tau_2)]$ by a nonlinear least-squares method with convolution of the instrument response function using in-house software as described previously.[27] Errors in lifetimes and percent contributions are estimated to be ±10%. All complexes were purified by HPLC prior to luminescence measurement using a Dynamax 300 Å C18 reverse-phase column (Rainin) on a Hewlett-Packard 1100 HPLC (95% 30 mM NH_4-OAc/5% acetonitrile to 100% acetonitrile over 60 min). Samples consisted of 10 μM racemic metal complex, 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. For determining excited-state lifetimes of metal complexes bound to DNA, 1 mM nucleotides ct-DNA was present. In luminescence quenching studies, samples contained, in addition, 80 μM $Ru(NH_3)_6^{3+}$ or 100 μM $Rh(phi)_2(dmb)^{3+}$ (phi = 9,10-phenanthrenequinone diimine; dmb = 4,4′-dimethyl-2,2′-bipyridine). The luminescence traces of the complexes in water were fit to a monoexponential function ($C_1 = 1$).

Determination of Binding Constants to DNA. Luminescence titrations on an ISS-K2 fluorometer were performed to determine affinity constants for the ruthenium complexes with ct-DNA. Ct-DNA, ranging from 10^{-7} to 10^{-3} M, was titrated into solutions containing racemic metal complex, 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. For each metal complex, multiple experiments were conducted at a constant metal concentration ranging from 0.25 to 10 μM. An excitation wavelength of 450 nm was used, and total luminescence intensity was recorded from 500 to 800 nm and corrected for dilution. The fraction of complex bound to ct-DNA was calculated from the equation $C_B = C_T[(F - F_F)/(F_B - F_F)]$, where C_T is the total concentration of metal complex, F the observed luminescence intensity, at a given ct-DNA concentration, F_F the intensity of unbound complex, and F_B the intensity of fully bound complex. Binding data was analyzed in the form of Scatchard plots.[28]

Determination of Enantioselectivity. A double stranded DNA cellulose (Sigma) column was rinsed with 20 mM sodium phosphate, 10 mM NaCl, pH 7.85 buffer to remove unbound DNA. Then 100 μL of 100 μM *rac*-$Ru(bpy)_2(dpq)^{2+}$ or *rac*-$Ru(bpy)_2(dpqC)^{2+}$ was loaded onto the column and eluted with 1 mL of buffer. Following this elution, 1 mL of 5 M NaCl was loaded onto the column and the eluant collected. Circular dichroism measurements were performed on both fractions using a AVIV circular dichroism spectrometer 62A DS.

Oligonucleotide Synthesis. Oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer, using standard phosphoramidite chemistry.[29] DNA was synthesized with a 5′-dimethoxy trityl (DMT) protecting group and was purified by HPLC using a Dynamax 300 Å C18 reverse-phase column (Rainin) on a Hewlett-Packard 1100 HPLC (95% 30 mM NH_4OAc/5% acetonitrile to 84% 30 mM NH_4OAc/16% acetonitrile over 25 min). The DMT group was removed by incubation with 80% glacial acetic acid for 12 min at ambient temperature and then repurified by HPLC (100% 30 mM NH_4OAc to 75% 30 mM NH_4OAc/25% acetonitrile over 40 min). Quantification was done on a Beckman DU 7400 spectrophotometer using the ϵ_{260} values estimated for single stranded DNA.[30]

Ruthenium-tethered 17-mer oligonucleotides were prepared as described previously[31] and were purified on a Dynamax 300 Å C18 reverse-phase column (Rainin) on a Hewlett-Packard 1050 HPLC (85% 30 mM NH_4OAc/15% acetonitrile to 75% 30 mM NH_4OAc/25% acetonitrile over 40 min). The ruthenium-conjugated oligonucleotides were characterized by mass spectrometry and quantitated using the following extinction coefficients: Ru(phen)(bpy′)$(dppz)^{2+}$ modified oligonucleotides $\epsilon_{432} = 19000\ M^{-1}\ cm^{-1}$;

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Table 1. Electrochemical Data[a] and $E_{0/0}$[b] for Ruthenium Complexes

complex	metal centered (*2+/+)	metal centered (3+/2+)	$E_{0/0}$	ligand centered L/L·⁻	ligand centered bpy_1·⁻	ligand centered bpy_2·⁻
$[Ru(bpy)_2(dppz)]^{2+}$ [c]	1.51	1.57	2.24	−0.73	−1.15	−1.39
$[Ru(bpy)_2(dppx)]^{2+}$	1.44	1.55	2.25	−0.81	−1.17	−1.39
$[Ru(bpy)_2(dpq)]^{2+}$ [c]	1.17	1.47	2.21	−1.04	−1.33	−1.48
$[Ru(bpy)_2(dpqC)]^{2+}$	1.21	1.45	2.28	−1.07	−1.27	−1.47
$[Ru(bpy)_3]^{2+}$ [c]	0.98	1.49	2.10	−1.12	−1.28	−1.51
$[Ru(phen)_3]^{2+}$ [c]	1.08	1.54	2.18	−1.10	−1.25	−1.42

[a] In dry CH_3CN with 100 mM tetrabutylammonium hexafluorophosphate; scan rate 100 mV/s. For all complexes examined, oxidation waves are reversible, and reduction waves are irreversible. Potentials are reported in volts vs NHE, and uncertainty is estimated to be ±50 mV. [b] Excitation and emission spectra were obtained in CH_3CN and the point of intersection, after normalization, was taken as $E_{0/0}$. $E_{0/0}$ values were obtained from the luminescent triplet excited state and are therefore underestimates of the actual values. [c] In agreement with literature values.[33,34]

$Ru(bpy)_2(bpy')^{2+}$ modified oligonucleotides $\epsilon_{453} = 21000\ M^{-1}\ cm^{-1}$.

Assay of Oxidative DNA Damage. For experiments conducted using noncovalently bound ruthenium, single strands containing the guanine doublet site were 5′-^{32}P end-labeled using standard protocols[32] and annealed to the complementary strand in an aerated buffer of 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. Oligonucleotide duplexes (8 μM) containing 16 μM racemic metal complex and 160 μM $Ru(NH_3)_6^{3+}$ as an electron accepting quencher were irradiated at 450 nm with a 1000 W Hg/Xe lamp equipped with a monochromator. Irradiation times varied from 0 to 30 min. After irradiation, samples were treated with 10% piperidine at 90 °C for 30 min, dried, and electrophoresed through a 20% denaturing polyacrylamide gel. The extent of damage was quantitated by phosphorimagery (ImageQuant).

For experiments conducted using ruthenium-tethered oligonucleotides, single strand complements to the ruthenium-modified oligonucleotides were 5′-^{32}P end-labeled as described[32] and annealed in an aerated buffer of 35 mM Tris-HCl, 5 mM NaCl, pH 8.0. Oligonucleotide duplexes (2.5 μM) with 25 μM MV^{2+} as an electron accepting quencher were irradiated for 5 min at 432 nm using a 1000 W Hg/Xe lamp equipped with a monochromator. After irradiation, samples were treated with 10% piperidine at 90 °C for 30 min, dried, and electrophoresed through a 20% denaturing polyacrylamide gel. The extent of damage was quantitated by phosphorimagery (ImageQuant).

Results

Redox Characteristics of Metal Complexes. The reversible oxidation waves corresponding to the Ru(III)/Ru(II) couples are observed between 1.45 and 1.57 V (Table 1). According to these values, all of the Ru(III) complexes, once generated *in situ* by flash/quench, are capable of oxidizing guanines in the DNA duplex. The electrochemical reduction of these complexes is ligand-based, where the first reduction is centered on the dppz (or dppz derivative) and the subsequent reductions involve the ancillary ligands.[35] As can be seen in Table 1, the first reduction wave can be tuned by derivatizing the dppz ligand, which is inherently easy to reduce because of its aromatic size, providing a large area for charge delocalization. Adding methyl groups at the 7 and 8 positions of dppz, yielding dppx, donates electron density toward the ring system, resulting in a ligand that is ∼80 mV harder to reduce. Removing the terminal ring of dppz, yielding dpq, produces a ligand which is less aromatic and ∼310 mV more difficult to reduce. Along a similar line, removing the aromaticity of the terminal ring from dppz, yielding dpqC, again results in loss of aromaticity and a ligand which is ∼340 mV harder to reduce.

Values for $E_{0/0}$ are also presented in Table 1. The values presented allow the calculation of excited state reduction potentials of the complexes (*2+/+), which is a critical parameter when considering guanine oxidation directly from the excited state. On the basis of the excited-state redox potentials of these complexes, the ruthenium complexes with dppz and dppx ligands possess excited-state potentials that should be capable of guanine oxidation.

Luminescence Characteristics in the Absence and Presence of DNA. Table 2 shows the excited-state lifetimes along with their emission maxima obtained in water, with ct-DNA, and in the presence of oxidative quenchers. As described earlier, $Ru(bpy)_2(dppz)^{2+}$ and $Ru(bpy)_2(dppx)^{2+}$ behave as luminescent "light switches"; no detectable luminescence is apparent in aqueous solution, but upon intercalation, the phenazine nitrogens are protected from aqueous quenching, and the complexes emit.[37] Luminescence decay traces for the dppz and dppx complexes bound to DNA show biexponential decays in emission, which we have assigned to two general orientations (side-on and head-on) for intercalation of the complexes into the duplex;[38] these orientations are supported also by NMR results.[39]

In contrast to $Ru(bpy)_2(dppz)^{2+}$ and $Ru(bpy)_2(dppx)^{2+}$, the complexes $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ do not show "light switch" behavior. Altering the electronic structure of the dppz ligand to yield dpq and dpqC results in complexes that emit in aqueous solvents in the absence of DNA. However, both $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ display 2−5-fold luminescence enhancements upon the addition of DNA. These complexes also display biexponential decays in emission when bound to DNA. These luminescence characteristics in the presence of DNA are reminiscent of those seen earlier with $Ru(phen)_3^{2+}$ and derivatives.[40,41]

In the presence of the oxidative quencher $Ru(NH_3)_6^{3+}$, all the DNA-bound ruthenium complexes examined display

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Table 2. Excited State Lifetimes for *rac*-Ruthenium Complexes (in Nanoseconds)[a]

complex	H_2O	ct-DNA	ct-DNA 80 μM $Ru(NH_3)_6^{3+}$ [c]	ct-DNA 100 μM $Rh(phi)_2(dmb)^{3+}$ [c]	emission max (nm)
$Ru(bpy)_2(dpq)^{2+}$	195 ± 11	1094 ± 39 (93%)	549 (93%)	862 (47%)	636
		47 ± 4 (7%)	217 (7%)	167 (53%)	
$Ru(bpy)_2(dpqC)^{2+}$	415 ± 26	965 ± 84 (90%)	532 (15%)	672 (47%)	609
		68 ± 5 (10%)	205 (85%)	121 (53%)	
$Ru(bpy)_2(dppz)^{2+}$ [d]	*b*	450 ± 32 (35%)	207 (20%)	115 (21%)	627
		50 ± 5 (65%)	42 (80%)	14 (79%)	
$Ru(bpy)_2(dppx)^{2+}$	*b*	475 ± 44 (39%)	187 (48%)	274 (52%)	619
		137 ± 9 (61%)	53 (52%)	30 (48%)	
$Ru(bpy)_3^{2+}$ [e]	406	420			

[a] Samples consisted of 10 μM racemic metal complex, 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. Lifetimes in water alone and ct-DNA are given as averages of three replicates. When present, ct-DNA concentration was 1 mM nucleotides. In fluorescence quenching studies, samples contained, in addition, 80 μM $Ru(NH_3)_6^{3+}$ or 100 μM $Rh(phi)_2(dmb)^{3+}$ (phi = 9,10-phenanthrenequinone diimine; dmb = 4,4′-dimethyl-2,2′-bipyridine). The luminescence traces of the complexes in water alone were fit to a monoexponential function ($C_1 = 1$). [b] Faster than instrument response. [c] Uncertainties estimated to be ±10%. [d] Corresponds to literature value.[36,40] [e] Literature value.[40]

Table 3. DNA Binding Properties for Ruthenium Complexes[a]

complex	K_b, M^{-1}	metal/nucleotides
$Ru(bpy)_2(dppz)^{2+}$ [b,c]	$>10^6$	
$Ru(bpy)_2(dppx)^{2+}$	8.8 (0.3) × 10^6	1/3
$Ru(bpy)_2(dpq)^{2+}$	5.9 (0.2) × 10^4	1/8
$Ru(bpy)_2(dpqC)^{2+}$	8.5 (0.2) × 10^4	1/4

[a] Ct-DNA, ranging from 10^{-7} to 10^{-3} M, was titrated into solutions containing 0.25−10 mM racemic metal complex, 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. An excitation wavelength of 450 nm was used, and total luminescence intensity was recorded from 500 to 800 nm and corrected for dilution. [b] Literature value.[16] [c] Values for $Ru(phen)_2(dppz)^{2+}$ are reported to be $\sim10^7$ in 50 mM NaCl, 1 mM sodium cacodylate, pH 7 and $\sim10^6$ in 5 mM Tris-HCl, 50 mM NaCl, pH 7.1.[17]

shorter excited-state lifetimes indicative of dynamic quenching by the groove-bound ruthenium hexammine. The longer lived component is assigned as the DNA-bound species; in the presence of quencher, this excited-state lifetime is shortened, resulting in two shorter lived components. $Rh(phi)_2(dmb)^{3+}$ has been shown to intercalate into DNA via the phi ligand and quench the emission of intercalating ruthenium complexes effectively via DNA mediated charge transport.[36] Quenching by $Rh(phi)_2(dmb)^{3+}$ was observed with all the ruthenium complexes examined, suggesting the intimate association of all of the ruthenium complexes with DNA.

Binding Affinities Determined through Luminescence Titration and Support for an Intercalative Binding Mode. Spectroscopic titrations of the ruthenium complexes with ct-DNA were carried out over a range of metal concentrations. Table 3 shows the results obtained through Scatchard analyses of the data. As expected, the dppz and dppx complexes possess intercalative binding affinities for DNA that are significantly higher than those for the dpq and dpqC complexes.

Because of the low binding affinities found for the dpq and dpqC complexes, additional experiments were carried out to probe whether binding to DNA by these complexes was primarily through an intercalative binding mode. Earlier studies with $Ru(phen)_3^{2+}$ indicated a mixture of binding

Table 4. Luminescence Polarization Data for Ruthenium Complexes[a]

	medium			
complex	buffer	ct-DNA	60% glycerol	ct-DNA/60% glycerol
$[Ru(bpy)_3]^{2+}$	−0.0004	0.005	0.001	0.006
$[Ru(phen)_3]^{2+}$ [b]	−0.001	0.006	0.002	0.027
$[Ru(bpy)_2(dpq)]^{2+}$	−0.003	0.012	0.003	0.045
$[Ru(bpy)_2(dpqC)]^{2+}$	−0.003	0.017	0.003	0.060
$[Ru(bpy)_2(dppx)]^{2+}$		0.025		0.061
$[Ru(bpy)_2(dppz)]^{2+}$		0.029		0.070

[a] Samples consisted of 10 μM racemic metal complex in 20 mM sodium phosphate, 10 mM NaCl, pH 7.85. When present, ct-DNA concentration was 1 mM nucleotides, and glycerol samples contained 60% glycerol by volume. Samples were irradiated at 450 nm and emission was monitored at 610 nm using a 495 nm cutoff filter. Uncertainties are estimated to be ±5%. [b] Comparable to literature values.[40]

modes.[40,42,43] Values for luminescence polarization are shown in Table 4. In buffer and 60% glycerol, there is no significant polarization observed for the complexes. In the presence of ct-DNA, there is an increase in polarization observed for all of the complexes, while the highest values of polarization are obtained in the presence of both ct-DNA and 60% glycerol. For these samples containing both ct-DNA and glycerol, the luminescence polarization values for $Ru(bpy)_3^{2+}$ and $Ru(phen)_3^{2+}$ are smallest, while $Ru(bpy)_2(dppz)^{2+}$ and $Ru(bpy)_2(dppx)^{2+}$ display the largest degree of luminescence polarization. Certainly, the values for the luminescence polarization depend in part on the luminescent lifetimes of the complexes; hence, all values are expected to be significantly lower than those for fluorescent intercalators such as ethidium (20 ns excited-state lifetime).[44] Given that the bound excited state lifetimes for the dpq and dpqC complexes are in the microsecond range, the values obtained indicate that, despite the low polarization values, these complexes are held in a relatively rigid environment bound to DNA. The values obtained therefore are consistent with intercalative binding.

The intercalative binding mode was also probed through measurements of enantioselectivity associated with binding

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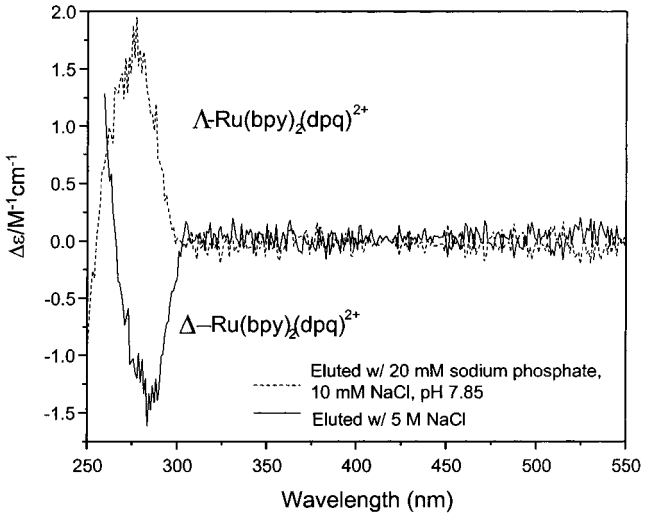

Figure 2. Circular dichroism for $Ru(bpy)_2(dpq)^{2+}$ eluted from DNA cellulose column with 20 mM sodium phosphate, 10 mM NaCl, pH 7.85 (dashed) and that eluted later with 5 M NaCl (solid). Configurations were assigned on the basis of literature spectra.[39]

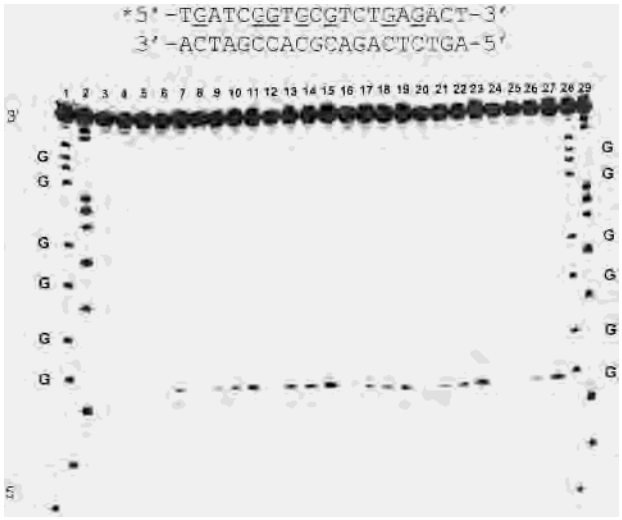

Figure 3. Oxidative damage to DNA via flash/quench method. Shown at the top is the sequence of oligonucleotides used for electrophoresis experiments. The double guanine site has been underlined indicating site with the lowest oxidation potential; single guanines have also been underlined as these sites are susceptible to $^1O_2$ damage. Site of ^{32}P labeling is indicated by *. Shown at the bottom is the autoradiogram after oxidation of the oligonucleotide by $Ru(bpy)_3^{2+}$ in lanes 4−7; oxidation by $Ru(phen)_3^{2+}$ in lanes 8−11; and oxidation by $Ru(bpy)_2(L)^{2+}$ (L = dppz, dppx, dpq, dpqC) in lanes 12−27, respectively, using the flash/quench technique for increasing periods of time of irradiation: 0, 5, 10, 30 min within each series. Samples contain 8 μM oligonucleotide, 16 μM metal, and 160 μM $Ru(NH_3)_6^{3+}$. Lanes 1, 28 and 2, 29 show the damage patterns after Maxam−Gilbert sequencing reactions A + G and C + T, respectively. Lane 3 shows the damage pattern after irradiation for 30 min of the oligonucleotide in the presence of quencher but absence of metal.

to DNA. Right-handed metal complexes bound through intercalation are favored in binding to the right-handed helix whereas the left-handed isomer is generally favored for a groove-bound mode with B-form DNA.[45] Enantioselectivity in DNA binding was probed by examining fractions eluted from a DNA cellulose column.[46] The circular dichroism spectra obtained for the fractions collected from the DNA cellulose column for $Ru(bpy)_2(dpq)^{2+}$ are shown in Figure 2. Spectra for $Ru(bpy)_2(dpqC)^{2+}$ showed identical patterns. Comparison with literature spectra[39] indicates that the less favored isomer, eluting with lower salt concentrations, corresponds to the Λ-enantiomer while the Δ-enantiomer, eluting with 5 M NaCl, binds preferentially to DNA. These data are therefore also consistent with intercalative binding.

Oxidative Damage by Noncovalently Bound Ruthenium Complexes. Ruthenium(III) complexes, generated *in situ* using the flash/quench technique, effectively damage DNA via hole transport chemistry followed by irreversible reaction of the guanine radical produced. Figure 3 shows the oligonucleotide sequence employed for these experiments as well as the results for the family of ruthenium complexes tested. It is evident that the damage obtained for all complexes resides solely on the 5′-G of the 5′-GG-3′ guanine doublet. This site is considered to be that of lowest oxidation potential within the oligonucleotide on the basis of empirical and theoretical studies,[14,15] and damage at the 5′-G of 5′-GG-3′ sites is generally taken as a hallmark of electron-transfer damage.[9] All of the ruthenium complexes examined produce this characteristic damage pattern, and increasing irradiation time leads to increased amounts of damage. Figure 4 shows the comparison of efficiencies for the different complexes.

Figure 5 shows the oxidative damage obtained after piperidine treatment of the oligonucleotides irradiated with

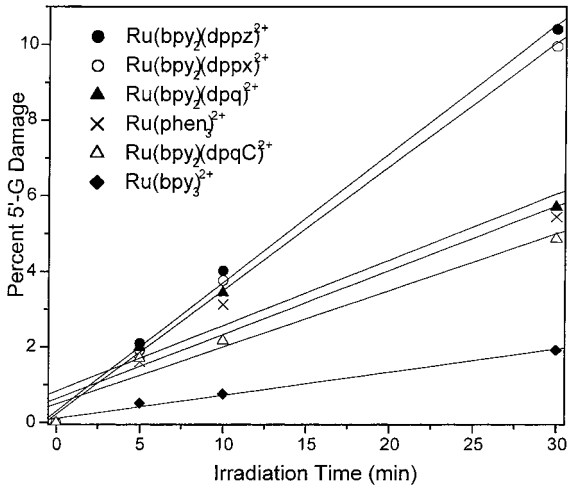

Figure 4. Plot of 5′-G damage versus irradiation time for family of ruthenium complexes showing relative efficiencies of oxidative damage.

the ruthenium complexes but in the absence of quencher. This damage is strikingly different from that in Figure 3, in that here damage is observed at all guanines with little sequence preference. This damage is not consistent with an electron-transfer reaction but is instead consistent with damage owing to reaction with singlet oxygen, formed by sensitization of the excited ruthenium complexes.[47,48] Singlet

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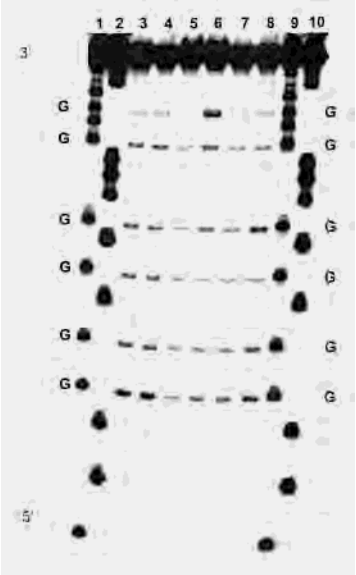

Figure 5. Oxidative DNA damage via singlet oxygen sensitization. Autoradiogram after denaturing polyacrylamide gel electrophoresis of ^{32}P-5′-TGATCGGTGCGTCTGAGACT-3′ after oxidation of the oligonucleotide by the family of ruthenium complexes, in the absence of quencher. Samples contain 8 μM oligonucleotide and 16 μM metal, after 30 min of irradiation at 450 nm. Lanes 1, 9 and 2, 10 show damage patterns after Maxam−Gilbert sequencing reactions A + G and C + T, respectively. Lanes 3−8 show damage pattern after oxidation of the oligonucleotide by $Ru(bpy)_3^{2+}$, $Ru(phen)_3^{2+}$, and $Ru(bpy)_2(L)^{2+}$ (L = dppz, dppx, dpq, dpqC), respectively.

oxygen reacts preferentially with guanines,[49] and the slight variations in base damage along the oligomer observed probably reflect preferences in the sites of Ru(II) binding and/or differences in the accessibility of guanine to diffusion of molecular oxygen.

Oxidative Damage by Covalently Bound Ruthenium Complexes. To probe the importance of intercalation to oxidative damage by long-range charge transport most directly, we compared oxidative damage patterns for $Ru(bpy)_2(bpy')^{2+}$ and $Ru(phen)(dppz)(bpy')^{2+}$ covalently bound to DNA. The bpy complex shows no intercalative interaction with the duplex while the dppz complex binds avidly by intercalation. By tethering the two complexes to the DNA duplex, one can therefore distinguish the effects of intercalation from simply a low association with the helix.

Figure 6 shows the results. As is evident, there is no guanine damage on the duplex containing the covalently bound $Ru(bpy)_2(bpy')^{2+}$ (lane 14) just as there is no guanine damage in the case of noncovalently bound $Ru(bpy)_2(bpy')^{2+}$ when the ratio of oligonucleotide to metal is 1:1 (lane 10). It is noteworthy that significant damage at all guanines is evident at a higher concentration of noncovalent $Ru(bpy)_2(bpy')^{2+}$ (lane 8). We attribute this oxidation to direct association of the ruthenium complex with the guanine site. Also, for comparison, the expected long-range guanine oxidation is observed with both the covalently (lane 12) and noncovalently bound (lanes 4, 6) dppz derivatives of ruthenium. Interestingly, in the case of noncovalently bound $Ru(bpy)_2(dppz)^{2+}$, damage is also observed at the adenine

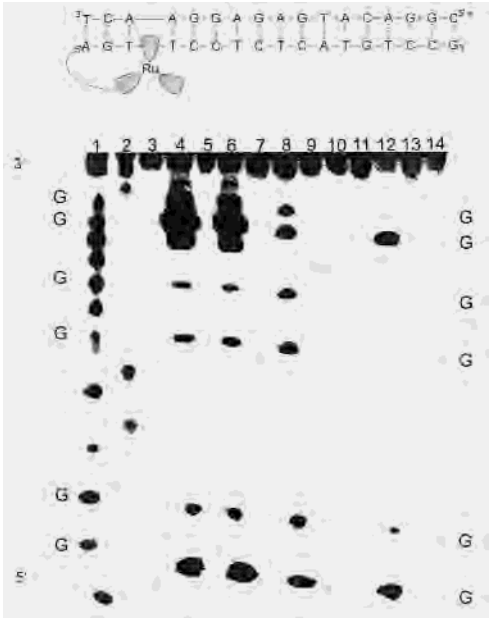

Figure 6. DNA damage by covalent versus noncovalent ruthenium complexes. Shown at the top is the ruthenium−oligonucleotide conjugate used in covalently tethered experiments. Site of ^{32}P labeling is indicated by *. Shown at the bottom is the autoradiogram of the ruthenium−oligonucleotide conjugate after oxidation by noncovalent and covalent bpy and dppz complexes. Samples contain 2.5 μM ruthenium−oligonucleotide and 25 μM MV^{2+} for covalently bound experiments. Noncovalently bound experiments utilized 2.5 μM oligonucleotides, 25 μM MV^{2+}, and indicated metal to DNA ratio. All irradiations were at 432 nm for 5 min. Lanes 1 and 2 show damage pattern after Maxam−Gilbert sequencing reactions A + G and C + T, respectively. Lanes 3, 4 and 5, 6 show damage pattern for oxidation of the oligonucleotide by $Ru(bpy)_2(dppz)^{2+}$, 4:1 and 1:1 metal to DNA, respectively, in absence of light and after irradiation. Lanes 7, 8 and 9, 10 show damage pattern for oxidation by $Ru(bpy)_2(bpy')^{2+}$, 4:1 and 1:1 metal to DNA, respectively, in absence of light and after irradiation. Lanes 11 and 12 show damage pattern after oxidation by covalently bound $Ru(phen)(dppz)(bpy')^{2+}$, in absence of light and after irradiation, respectively. Lanes 13 and 14 show damage pattern by covalently bound $Ru(bpy)_2(bpy')^{2+}$, in absence of light and after irradiation, respectively.

5′ to the proximal double guanine site. This adenine, as part of a purine tract, may be particularly susceptible to oxidative damage. Control experiments confirm that the long-range guanine damage occurs intraduplex and is not a result of metal intercalation into DNA other than that to which it is covalently tethered.[7]

Discussion

Intercalative Binding by the Family of Ruthenium Complexes. The data shown here provide support for intercalative binding by the full family of ruthenium complexes. The binding affinity and extent of intercalation appear to correlate with the expanse of the ligand available for stacking between the DNA base pairs.[50]

A series of luminescence measurements were useful in characterizing the intercalative interaction. Comparable studies were carried out more than a decade ago to characterize the interaction of $Ru(phen)_3^{2+}$ and derivatives with DNA,[40] and analogous studies have been carried out more recently in characterizing various phenazine derivatives.[51] As seen

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earlier, the dppz and dppx complexes display no detectable luminescence in aqueous solution yet exhibit intense luminescence in the presence of DNA.[37,38] This effect has been attributed to the deep intercalation of the phenazine moiety within the base stack, so as to protect the phenazine nitrogen atoms from water. In the case of dpq and dpqC complexes, extensive NMR studies have been used to characterize interactions with DNA,[18,19] but luminescence properties have not been previously explored. Lacking the phenazine moiety, luminescence for $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ is observed in water even in the absence of DNA. Nonetheless, the complexes do show 2−5-fold luminescence enhancements in the presence of DNA, consistent with partial intercalation. In the case of $Ru(phen)_3^{2+}$, we had attributed comparable levels of luminescence enhancement to partial intercalation, rigidifying the complex within the helix, decreasing vibrational modes of relaxation.[40] We also observe that, similarly to $Ru(bpy)_2(dppz)^{2+}$ and $Ru(bpy)_2(dppx)^{2+}$, the excited states of $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ are quenched by groove-bound quenchers such as $Ru(NH_3)_6^{3+}$ and more efficiently by intercalating $Rh(phi)_2(dmb)^{3+}$, pointing to an intimate association with the DNA helix. The polarization of luminescence of $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ is comparable to that of $Ru(bpy)_2(dppz)^{2+}$, despite their relatively long excited-state lifetimes, and not to those of groove-bound $Ru(bpy)_3^{2+}$. Again, the polarization results support a rigid association of the complexes on the helix.

We also utilized measurements of enantioselectivity to distinguish the association with the right-handed helix by intercalation versus groove-binding. We had seen earlier that owing to symmetry and steric constraints, for an intercalative interaction, where the complex resembles a base pair in stacking within the helix, a right-handed Δ-configuration is favored for binding to right-handed duplex DNA; in contrast, for groove-binding against the right-handed helix, a complementary Λ-configuration is favored.[45] Enantioselectivity experiments clearly show for $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ that it is the Δ-enantiomer that is preferred in binding to the right-handed duplex, consistent with intercalation.

On the basis of binding affinity data, luminescence measurements, and the aromatic expanse of the intercalating ligand, there are three distinct classes within this family of ruthenium complexes. The first is composed of the dppz and dppx complexes; these display the highest binding affinities, reflecting deep intercalation within the helix. The second group contains complexes that bind less avidly to DNA, presumably because of decreased aromatic size of the intercalating ligand, $Ru(bpy)_2(dpq)^{2+}$, $Ru(bpy)_2(dpqC)^{2+}$, and $Ru(phen)_3^{2+}$. $Ru(phen)_3^{2+}$ had been shown to bind to DNA through a mixture of binding modes.[40] Because of the hydrophobicity associated with dpq and dpqC, a groove-binding association seemed reasonable to consider as a predominant mode of association. While a mixture of binding modes may result for these more weakly bound complexes, just as was seen earlier for $Ru(phen)_3^{2+}$, certainly the results described here are consistent with the presence of an intercalative interaction. The third class contains $Ru(bpy)_3^{2+}$; earlier studies had shown only the electrostatic association of this complex with DNA.[40]

Different Modes of Reactivity. Photoactivation of the ruthenium complexes bound to DNA leads to two distinct routes for oxidative damage, and these routes may be distinguished by the pattern of reactivity along the duplex. When this family of ruthenium complexes is irradiated in the presence of DNA, but absence of quencher, damage is observed at all guanines. This damage is consistent with singlet oxygen-mediated chemistry and is dependent on the excited state lifetime as well as binding affinity of the ruthenium complex. Singlet oxygen has been shown to react preferentially with guanines along the helix.[49] Because the reaction depends on the diffusion of $^1O_2$ from the site of sensitization to guanine, if the binding of the ruthenium sensitizer is nonspecific, then reaction at all guanines on the helix is expected. This reaction at all guanines is essentially what we observe for all of the complexes, and thus, the pattern reflects also the nonspecific association of the family of complexes with DNA.

When these ruthenium complexes are utilized in a flash/quench scheme, damage at the 5′-G of a 5′-GG-3′ guanine doublet is observed, the hallmark of DNA-mediated charge transport damage. This damage also depends on the excited-state lifetime of the metal complex. With this chemistry, however, a diffusible intermediate is not involved in generating the guanine radical. Instead, the reactivity depends on redox potentials, that of the ruthenium(III/II) couple and that of the guanine. It is the oxidation potential of the 5′-G of 5′-GG-3′ sites that appears to be lowest,[14,15] and hence the signature damage at 5′-G's. It is noteworthy that the guanine oxidation products are expected to be similar for singlet oxygen and charge transport damage,[24] although the products have not been characterized here. Also important to note is that because charge-transfer damage can arise from a distance, the site of reactivity need not reflect the site of ruthenium binding.

Interestingly, a third mode of reactivity would be direct DNA oxidation from the Ru(II) excited state. From the excited state reduction potentials, both the dppz and dppx complexes appear to have the proper driving force to oxidize guanines from the excited state. However, this damage is not observed. A possible explanation comes from an examination of the Ru(II) excited state. The excited state results from a metal-to-ligand charge transfer, which is directed to the dppz or dppx ligand. When these complexes intercalate into DNA, the ligand which is in intimate contact with the π-stack and would ultimately accept an electron from guanine therefore possesses additional electron density in the excited state. Most likely, then, because of the direction of the charge transfer, the driving force for this reaction is significantly less than expected.

Direct Correlation between Intercalation and DNA Charge Transfer. For the family of ruthenium complexes

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examined here, it is evident that there is a direct correlation between binding affinity and efficiency of damage as a result of DNA-mediated charge transfer. Tighter intercalative binding results in greater amounts of oxidative DNA damage. Complexes that possess large aromatic ligands intercalate more avidly than those with less aromatic surface area and also display greater amounts of oxidative damage. Thus, $Ru(bpy)_2(dppz)^{2+}$ and $Ru(bpy)_2(dppx)^{2+}$ show the highest extent of charge transport damage, despite not having particularly long excited-state lifetimes bound to DNA. $Ru(bpy)_3^{2+}$, although possessing a long excited-state lifetime,[40] shows no evidence for an intercalative association with DNA and shows little reactivity through charge transport chemistry. $Ru(bpy)_2(dpq)^{2+}$ and $Ru(bpy)_2(dpqC)^{2+}$ have intermediate levels of intercalative binding and show intermediate levels of oxidative damage through DNA charge transport.

The importance of intercalation is perhaps most directly illustrated in experiments comparing reactions of covalently bound ruthenium complexes. By tethering the complexes to the DNA duplex, one can distinguish the effects of intercalation from simply a low association with the helix. When the bpy complex is tethered to DNA, and thus linked to the helix, no DNA charge transfer damage from a distance results; damage is evident only at the site of ruthenium association. We attribute this lack of reactivity at distal positions to the lack of coupling of the ruthenium oxidant into the base pair stack. However, in the case of a tethered dppz complex, extensive damage is observed across the helix and at a site distant from ruthenium intercalation. It is noteworthy that at high enough metal concentrations guanine damage can be observed with noncovalent $Ru(bpy)_3^{2+}$; we attribute this damage to direct contact between guanine and $Ru(bpy)_3^{3+}$ generated in solution. Such reactivity by $Ru(bpy)_3^{3+}$ is precedented,[52] although no oxidative damage to DNA *from a distance* has been observed with $Ru(bpy)_3^{3+}$.

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More subtle variations in efficiency with degree of intercalative binding have also been seen. Differences in efficiency of guanine oxidation via DNA charge transport are observed for Δ- and Λ-enantiomers of Ru(II) and Rh(III) octahedral complexes.[36] The Δ-enantiomer can intercalate deeply into the base pair stack, avoiding steric interactions with the sugar−phosphate backbone of the right-handed helix, and provide efficient coupling between the oxidant and the π-stack. Decreased amounts of charge transport damage are observed with the Λ-enantiomers.

Intercalation therefore serves sensitively to modulate long-range oxidative DNA damage. Transient absorption experiments suggest that, as one might expect, the extent of intercalation correlates with the rate of hole injection. However, the data presented here do not allow us to distinguish between contributions to hole injection and charge migration, and indeed, coupling into the base pair may affect both steps in the charge transport process.

Implications and Conclusions. By studying a family of ruthenium complexes containing the dppz ligand and derivatives, the ability of the complex to intercalate into the DNA π-array has been found to affect directly the extent of DNA charge transport and resultant damage. Intercalation can lead to more effective coupling into the π-stack, resulting in more efficient hole injection and charge transport. These results require consideration in comparing reactions on DNA with different photooxidants. The source of charge injection into DNA is therefore a critical parameter in determining the extent of oxidative DNA damage from a distance.

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