DNA−Protein Cross-Linking from Oxidation of Guanine via the Flash−Quench Technique

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Abstract: The production of guanine radicals in DNA via the flash−quench technique is shown to cause the formation of covalent adducts between DNA and histone protein. In the flash−quench experiment, the DNA-bound intercalator $Ru(phen)_2dppz^{2+}$ (phen = 1,10-phenanthroline, dppz = dipyridophenazine) is excited with 442 nm light and quenched oxidatively by $Co(NH_3)_5Cl^{2+}$, methyl viologen (MV^{2+}), or $Ru(NH_3)_6^{3+}$ to produce $Ru(phen)_2dppz^{3+}$, a strong oxidant (+1.6 V) that can oxidize a nearby guanine base (+1.3 V). The guanine radical thus produced is vulnerable to nucleophilic attack and can react with amino acid side chains to form DNA−protein cross-links. Evidence for DNA−protein cross-linking was provided by the chloroform extraction assay, a filter binding assay, and gel electrophoretic analysis. After flash−quench treatment, pUC19 plasmid DNA undergoes a dramatic decrease in mobility that is reversed upon digestion with proteinase K, as seen by agarose gel electrophoresis. In polyacrylamide gel electrophoresis (SDS-PAGE) experiments, the histone protein shows similar mobility shifts. Cross-linking is observed with poly(dG-dC) and mixed sequence DNA, but not with poly(dA-dT), indicating that the reaction requires guanine bases. Measurements of emission quenching indicate that for a given quencher, the amount of cross-linking is correlated to the amount of quenching. When comparing different quenchers, however, the amount of cross-linking is inversely related to the amount of quenching and decreases in the order $Co(NH_3)_5Cl^{2+} > MV^{2+} > Ru(NH_3)_6^{3+}$. This trend in cross-linking correlates instead with the lifetime of the guanine radical measured by transient absorption spectroscopy, and suggests that the cross-linking reaction requires > 100 μs. These results demonstrate that the flash−quench technique is an effective approach for the study of covalent adducts between DNA and protein formed as a result of guanine oxidation, and suggest one possible fate for oxidatively damaged DNA in vivo.

Introduction

Oxidative damage to DNA is a major factor in aging and in many molecular diseases such as Parkinson's and Alzheimer's diseases, as well as cancer.[1] As the DNA base with the lowest oxidation potential,[2] guanine plays a special role because it has the greatest susceptibility to oxidative damage. Oxidation of guanine leads to formation of 7,8-dihydro-8-oxoguanine (8-oxo-G) and other damage products,[3] but such lesions are not the only fate for guanine radicals. DNA−protein cross-linking is another possible consequence of guanine oxidation, as will be described here.

Although oxidative damage to DNA is a subject of tremendous physiological relevance, the study of guanine radicals in DNA presents a challenge to researchers. Many techniques, including pulse radiolysis,[4] laser photolysis,[5] and type I photochemical oxidations,[6] have been shown to generate guanine radicals in solution. However, it is more difficult to form 1-electron-oxidized guanine *in double-stranded DNA*, where the stacked bases enjoy some protection from solvent-borne oxidants, without ionizing radiation[7] or photolysis by UV laser light.[8] While photosensitizers can create guanine radicals, there is typically considerable interference from $^1O_2$, since only a select few appear to oxidize G solely by a type I mechanism.[9] Nonetheless, some direct photooxidants have proven useful in guanine oxidation studies; thus far, these include phenanthrenequinone diimine (phi) complexes of rhodium(III),[10] naphthalamides,[11] cationic anthraquinones,[12] and riboflavin.[13] Other approaches profitably applied to guanine oxidation include the use of nucleosides with a sugar moiety modified at the C4′

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position,[14] electrochemical oxidation by $Ru(bpy)_3^{3+}$ [bpy = bipyridine],[15] and the flash−quench technique.[16,17]

Originally developed for the study of electron transfer in proteins,[18] the flash−quench technique has proven to be ideal for studying reactions of guanine radicals in DNA. In the flash−quench technique, a photosensitive intercalator is excited by visible light and converted to a strong oxidant by an oxidative quenching reaction; the oxidized intercalator can then remove an electron from a nearby guanine base. Dunn et al. first applied this technique, also called photochemical cosensitization, to demonstrate selective damage at guanine bases in DNA upon quenching of excited ethidium by methyl viologen.[19] Using $Ru(phen)_2dppz^{2+}$ as the photosensitive intercalator and $Ru(NH_3)_6^{3+}$ as the quencher, we detected the guanine radical in poly(dG-dC) by transient absorption spectroscopy[16] and showed its UV−visible spectrum to be similar to that of the deprotonated neutral form, as reported by Candeias and Steenken.[4a] The formation of the neutral guanine radical, hereafter denoted simply as the guanine radical, was shown to occur rapidly (<50 ns).[16] The extent of permanent damage at G was tuned by the choice of quencher, and was shown to decrease in the order $Co(NH_3)_5Cl^{2+}$ > methyl viologen > $Ru(NH_3)_6^{3+}$, indicating that the amount of damage is controlled by the reactivity of the reduced quencher. Later work using the flash−quench technique[17,20] and other approaches[10,12,14] has demonstrated that the guanine radical is highly mobile in DNA, able to migrate up to 200 Å from the site of damage. Allowing both spectroscopic detection of reactive intermediates and observation of permanent damage products, the flash−quench technique is particularly well-suited for investigations of guanine oxidation chemistry.

DNA−protein cross-links, covalent adducts between DNA and protein, represent a major form of DNA damage.[21] These adducts are not as readily repaired as other DNA lesions[22] and can be lethal to the cell when appropriate repair mechanisms are compromised.[23] DNA−protein cross-links have been shown to result from a variety of sources, including cisplatin,[21] Ir(IV),[24] Ni(II),[25] Cr(VI),[26] phenanthroline-Cu(II),[27] Fe(II),[28] Fe(III) bleomycin,[27] organic carcinogens such as aldehydes[29] or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG),[30] peroxidized proteins,[31] magnetic fields,[32] ultraviolet light,[33] X-rays,[34] γ-rays,[35] and visible light with $^1O_2$ sensitizers.[36] Unfortunately, given the nonspecific nature of many of these reactions, there is little known about such lesions at the molecular level.

Oxidative damage of DNA can lead to the formation of DNA−protein cross-links,[3,24−27,36] and guanine is particularly vulnerable to cross-link formation, because it is the only base that can be oxidized photochemically by both type I and type II mechanisms.[6] The guanine cation radical can be hydrated en route to formation of 8-oxo-G,[8d,13,37] and the 1-electron-oxidized guanine base is susceptible to attack from other nucleophiles as well.[38,39] Given that DNA is typically bound to protein, and that proteins have many nucleophilic side chains, one likely consequence of guanine oxidation in vivo is the formation of DNA−protein cross-links. In well-characterized model systems, Morin and Cadet have shown that 1-electron-oxidized 2′-deoxyguanosine readily forms covalent adducts with nucleophiles.[38,39] Using benzophenone as a type I photosensitizer, they oxidized 2′-deoxyguanosine in solution and demonstrated cross-linking with both hydroxyl- and amino-based nucleophiles, illustrating that DNA−protein cross-links could be effected by reactions of guanine radical with Ser, Thr, and Lys. Morin and Cadet found that covalent bond formation with the nucleophile occurred at C8 of guanine. This result is noteworthy, given that this position should be at least somewhat accessible to agents bound in the major groove of DNA and that most DNA−protein interactions occur in the major groove of DNA.[40]

As an established method for selectively generating guanine radicals in DNA, the flash−quench technique shows particular promise for the study of DNA−protein adducts caused by guanine oxidation. In contrast to ionizing radiation,[41] the flash−quench technique produces a single reactive species on the DNA, which should impart some specificity to the cross-linking reaction and thereby limit the number of products. Moreover, the mobility of the electron hole allows for reaction between DNA and protein to occur far from the initial site of oxidation, so that the cross-linking agent need not interfere with the DNA−protein binding interaction.

Here, we introduce the flash−quench technique as a novel method for the induction of DNA−protein cross-links. This cross-linking reaction requires the presence of guanine bases, and the yield correlates with the amount of quenching, as expected for a process involving guanine radicals formed by the quenching event. The choice of quencher strongly influences

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the yield of cross-linking and the lifetime of the guanine radical, as determined by transient absorption spectroscopy, suggesting that the guanine radical must persist for longer than 100 μs for adducts to form. These results demonstrate the utility of the flash−quench technique as a way to study cross-links between DNA and protein that result from oxidative damage of guanines in DNA.

Experimental Methods

Materials. Isopentyl alchohol, sodium phosphate, sodium chloride, and sodium hydroxide were obtained from Mallinckrodt. Chloroform, ethidium bromide, hexaammineruthenium(III) chloride, pentaamminecobalt(III) chloride, and methyl viologen were from Aldrich. Ultrapure agarose was obtained from Gibco BRL. Urea was from ICN. Sodium dodecyl sulfate (SDS, BioRad) was of electrophoresis grade. [Ru(phen)$_2$dppz]Cl$_2$ was prepared as described previously.[42] Histone III-S from calf thymus was obtained from Sigma. pUC19 plasmid was from New England Biolabs. Proteinase K, sonicated calf thymus DNA (CT DNA), poly(dG-dC), and poly(dA-dT) were purchased from Amersham Pharmacia. The average lengths of the DNA polymers, as reported by the manufacturer, were ∼3000 base pairs (bp) for CT DNA, ∼750 bp for poly(dG-dC), and ∼1000 bp for poly(dA-dT). Prior to use, solutions of DNA polymers were exchanged into a buffer of 10 mM sodium phosphate (NaPi), 20 mM NaCl (pH 7) via multiple rounds of ultrafiltration using Centricon 30 microconcentrators (Amicon). Stock solutions were prepared utilizing the following extinction coefficients: $\epsilon_{276} = 0.460$ mM^{-1} cm^{-1} for Ru(NH$_3$)$_6^{3+}$, $\epsilon_{440} = 21.0$ mM^{-1} cm^{-1} for Ru(phen)$_2$dppz^{2+}, $\epsilon_{257} = 20.7$ mM^{-1} cm^{-1} for methyl viologen, $\epsilon_{260} = 6.60$ mM^{-1} cm^{-1} for calf thymus and pUC19 DNA, $\epsilon_{262} = 6.60$ mM^{-1} cm^{-1} for Poly(dA-dT), and $\epsilon_{254} = 8.40$ mM^{-1} cm^{-1} for Poly(dG-dC); concentrations of DNA are given in nucleotides (nuc). Co(NH$_3$)$_5$Cl$_3$ and histone were quantified by weight. Because the cobalt complex slowly decomposes in solution, fresh solutions were made prior to each set of experiments.

Preparation of Oligonucleotides. Oligonucleotide strands were synthesized on an ABI DNA/RNA synthesizer. Following initial purification by an oligo purification cartridge (OPC, Applied Biosystems) or by reversed-phase HPLC in the trityl-on form, the strands were then purified to homogeneity by reversed-phase HPLC in the trityl-off form. Oligonucleotide duplex was formed by heating a solution containing the strands in a 1:1 ratio to 90 °C, followed by slow cooling over several hours to room temperature.

Sample Irradiation. Samples (40−100 μL) containing Ru(phen)$_2$dppz^{2+} (10 μM), quencher (100 μM), DNA (1 mM nucleotides), and histone (250 μg/mL type III-S protein) in an aerated buffer of 10 mM NaPi and 20 mM NaCl (pH 7) were prepared. DNA was added last, and the samples were then kept under conditions of reduced light. Irradiation was carried out using either a 1000 W Xe arc lamp or a 1000 W Hg/Xe arc lamp as the light source. The Xe lamp beam was passed through an infrared water filter, a dichroic mirror (420−620 nm), a long pass ultraviolet filter (GG 355), a 442 nm interference filter, and a focusing lens to yield a final output at 442 nm of ∼5 mW. Access to the Hg/Xe lamp assembly was graciously supplied by Professor J. K. Barton; this lamp was outfitted similarly to the Xe lamp, except that it used a monochromator for wavelength selection and gave a final output of 2−3 mW at 442 nm.

Chloroform Extraction Assay. In this assay, noncovalent associations between DNA and protein are disrupted by dissociating conditions, and proteinaceous material is extracted from the samples with an organic solvent, leaving free DNA in the aqueous phase. Irradiated samples were adjusted to concentrations of 1% sodium dodecyl sulfate and 1 M NaCl. The solution was vortexed vigorously for 15 s, mixed with two volumes of chloroform:isopentyl alcohol (24:1), and vortexed again for 30 s. The two phases were separated by centrifugation at 6000 rpm for 15 min, and the 260 nm absorbance of the aqueous phase was measured by UV-spectroscopy, using a Hewlett-Packard HP8453 diode array spectrophotometer. The fraction of DNA−protein cross-linking was determined by using the equation below:

$$\text{fraction cross-linked} = [A(0) - A(t)]/A(0) \quad (1)$$

where $A(0)$ is the absorbance of the unirradiated control sample and $A(t)$ is the absorbance of a sample irradiated for time t.

Filter Binding Assay. This assay is based on the preferential binding of protein to nitrocellulose membranes. Irradiated samples were adjusted to a final concentration of 1.5 M NaCl/0.5 M urea and vortexed for 10 s. The samples (200 μL) were then passed through a nitrocellulose membrane filter (Millipore type HA, 2.5 cm), prewetted with water, by means of a vacuum sampling manifold (Millipore model 1225). The filters were washed twice with 1.5 M NaCl/0.5 M urea (300 μL). The amount of free DNA passed by the membrane was measured by means of UV-spectroscopy, and the fraction of DNA−protein cross-linking was determined by eq 1.

SDS-PAGE. An aliquot of the irradiated sample was mixed with H_2O and a loading buffer (NuPAGE LDS sample buffer, Novex) to give a sample containing 24 μg/mL protein. The samples (1.2 μg/well) were then analyzed by electrophoresis through a 10% polyacrylamide gel (Novex), with a running buffer of 50 mM Tris-MOPS, 1 mM EDTA, 0.1% SDS, pH 7.7 (Novex). At the completion of the run, the gel was fixed with a 4:5:1 water:methanol:acetic acid solution (Novex) and then stained with Coomassie dye (Colloidal Blue Kit, Novex). Images of the gels were obtained using a IS-1000 CCD camera (Alpha Innotech) or an HP 4C scanner (Hewlett-Packard), and were digitized using UN-SCAN IT software (Silk Scientific). When large DNAs were used, cross-linked material was unable to enter the gel, even at polyacrylamide concentrations as low as 3%. Thus, to ensure that the cross-linked material could enter the gel, this experiment was carried out with a 20-mer oligonucleotide duplex comprised of 5′-GCAATCGTGCG-TAGCAGAGC-3′ and its complement.

Agarose Gel Electrophoresis. In these experiments, pUC19 plasmid DNA was used. Irradiated samples were diluted 5× with buffer and adjusted to a concentration of 1% SDS, with bromophenol blue and xylene cyanol present as tracking dyes. Approximately 3 μg of DNA were added to each well of an 0.8% agarose gel. The gel was run at 70 V for ∼3 h at room temperature, in a buffer of 45 mM Tris-borate, 1 mM EDTA, pH 8.3 (National Diagnostics), with the use of a HE99 horizontal submarine apparatus (Hoefer). The bands were visualized by ultraviolet illumination after staining with ethidium, and images were obtained by using a polaroid documentation device (Fotodyne) or via an IS-1000 imaging system (Alpha Innotech).

Photophysical Measurements. Time-resolved luminescence and absorption measurements utilized the 480 nm output (1−2 mJ/pulse, Coumarin 480) of an excimer-pumped dye laser, as described elsewhere.[43] In titration experiments, individual samples were prepared for each quencher concentration. Emission of the ruthenium lumiphore was monitored at 610 nm, and the emission intensity was obtained by integrating under the luminescence decay curve. Luminescence lifetimes were obtained by fitting the decay curves to exponential functions by using in-house software. Stern−Volmer plots were used to obtain bimolecular quenching constants (k_q), according to eqs 2 and 3:

$$I_0/I = 1 + K_{SV}[Q] \quad (2)$$

$$k_q = K_{SV}/\tau_{avg} \quad (3)$$

where I_0 = emission intensity in the absence of quencher (Q), I = emission intensity at quencher concentration [Q], K_{SV} = the Stern−Volmer constant (obtained from plots of I_0/I vs [Q]) and τ_{avg} = the average emission lifetime in the absence of quencher.

Results and Discussion

Description of the Flash−Quench Technique. The flash−quench technique offers a simple way to produce guanine radical by using visible light to create a strong ground-state oxidant

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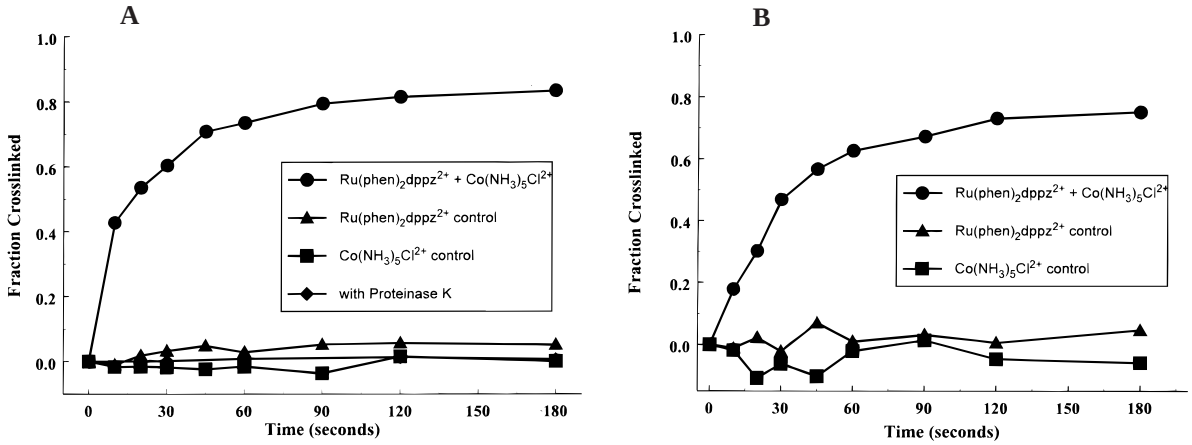
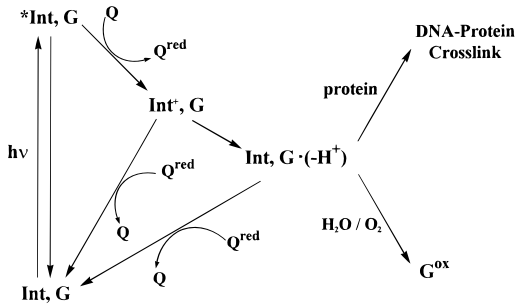


Figure 1. DNA−protein cross-linking detected by the chloroform assay (A) and a nitrocellulose filter binding assay (B). Shown are samples containing $Ru(phen)_2dppz^{2+}$, $Co(NH_3)_5Cl^{2+}$, CT DNA, and histone (circles), $Ru(phen)_2dppz^{2+}$, CT DNA, and histone (triangles), and $Co(NH_3)_5Cl^{2+}$, CT DNA, and histone (squares). Also shown (diamonds) in (A) are data for a sample treated with proteinase K (100 μg/mL for 1 h at 37 °C) prior to workup via the chloroform assay. Conditions: 10 μM $Ru(phen)_2dppz^{2+}$, 100 μM $Co(NH_3)_5Cl^{2+}$, 1 mM nucleotides sonicated CT DNA, and 250 μg/mL type III-S histone in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7. Irradiation was carried out at 442 nm at ~5.5 mW.

Scheme 1



on the DNA. The potent oxidant, created in situ by oxidative quenching of a photoexcited intercalator bound to DNA, removes an electron from guanine to make the guanine radical. In this method (Scheme 1), a ground-state intercalator, **Int**, is excited with visible light to form the excited state ***Int**, which can then transfer an electron to quencher **Q**. The oxidant thus formed, $\mathbf{Int}^+$, can either undergo back-electron transfer with reduced quencher $\mathbf{Q}^{red}$ or oxidize guanine, **G**. The resulting guanine radical is depicted as the neutral deprotonated from, **G•(-H$^+$)**, because of its low pK_a.[4a,44] This radical can either be repaired by electron transfer from $\mathbf{Q}^{red}$ or react further to yield permanent damage in the form of $\mathbf{G}^{ox}$ or a **DNA−protein cross-link**. In the experiments described here, **Int** represents $Ru(phen)_2dppz^{2+}$ and **Q** represents $Co(NH_3)_5Cl^{2+}$, MV^{2+} or $Ru(NH_3)_6^{3+}$. Given that $E°$ (Ru^{3+}/Ru^{2+}) = +1.6 V for $Ru(phen)_2dppz$[45] and that $E°$ (G^+/G) = +1.3 V,[2c] formation of the guanine radical by the flash−quench technique is favorable by ~0.3 V.

Detection of DNA−Protein Cross-Linking. For this investigation, we chose histone III-S, which is rich in basic residues and makes up part of the nucleosome core particle,[46] as a model DNA-binding protein. DNA−protein cross-links generated with the flash−quench technique were detected by using three different methods: chloroform extraction, filter binding, and gel electrophoresis. The chloroform extraction and filter binding assays monitor the disappearance of free DNA, while the agarose and polyacrylamide gel electrophoresis experiments allow one to follow the fate of the DNA and protein, respectively.

Figure 1**A** shows DNA−protein cross-linking detected by the chloroform extraction assay for samples containing $Ru(phen)_2dppz^{2+}$, $Co(NH_3)_5Cl^{2+}$, histone, and CT DNA. For the flash−quench samples containing all four reactants, the amount of cross-linking increases smoothly as the irradiation time increases, leveling off at ~85% at longer times. Over 50% of the DNA is cross-linked to protein within 20 s, and 83% of the DNA is cross-linked within 3 min of irradiation time. Control samples in which intercalator or quencher were omitted gave 5 and 0.1% cross-linked at the longest irradiation times, respectively. The fact that substantial cross-linking was seen only in samples with both intercalator and quencher is important and suggests that cross-linking from direct photooxidation and singlet oxygen sensitization is minimal under these conditions. Moreover, the cross-linking requires an association between DNA and protein, since bovine serum albumin, an acidic protein, does not exhibit cross-linking to DNA in the flash−quench experiment.[47]

A filter binding assay was also used to detect DNA−protein cross-links. As shown in Figure 1B, the amount of cross-linking in the flash−quench samples increases smoothly with increasing irradiation time. At 20 s, 30% of the DNA was cross-linked to protein, and 75% is cross-linked within 3 min of irradiation time. Again, control samples gave minimal amounts of cross-linking.

Agarose gel electrophoresis of pUC19 DNA was used to further demonstrate DNA−protein cross-linking via the flash−quench technique (Figure 2). The plasmid alone (lane 1) exhibits two bands, corresponding to the supercoiled form and the less mobile nicked form. A marked decrease in the mobility of the plasmid is observed when samples containing intercalator, quencher, histone, and DNA are irradiated with 442 nm light. As the irradiation time increases from 0 to 240 s (lanes 4−7), the main bands of the plasmid decrease in mobility and intensity, with considerable broadening apparent. By the longest irradiation

(44) Because we observed only the neutral deprotonated radical form in earlier flash−quench experiments (ref 16), this is the form depicted in the scheme. However, the guanine cation radical initially produced upon 1-electron oxidation can also be hydrated to form the 8-hydroxy-7,8-dihydroguanyl radical (see refs 8d and 13).

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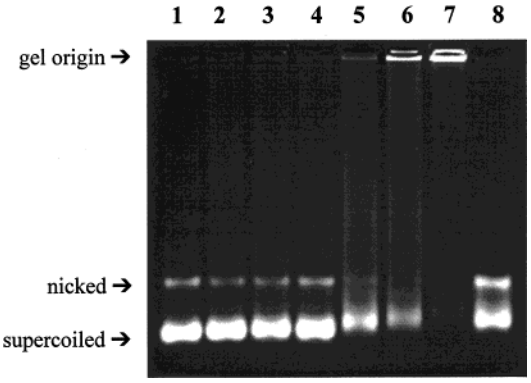

Figure 2. Cross-linking between pUC19 DNA and histone analyzed by agarose gel electrophoresis. Lane 1: DNA only, irradiated for 60 s. Lane 2: DNA + $Ru(phen)_2dppz^{2+}$ + histone, irradiated for 60 s. Lane 3: DNA + $Co(NH_3)_5Cl^{2+}$ + histone, irradiated for 60s. Lanes 4−7: DNA + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ + histone, irradiated for 0, 15, 30, 60 s, respectively. Lane 8: DNA + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ + histone, irradiated for 60 s, and treated with proteinase K as in Figure 1A prior to loading. Irradiated samples were diluted with water and loading buffer, and run on a 0.8% agarose gel at ∼70 V for 3 h, with ∼3 μg of DNA/well. Conditions for irradiation: same as in Figure 1, but with 2.7 mW 442 nm light and pUC19 DNA.

time, the pUC19 DNA is mostly confined to a tight band near the well. As shown in lane 8, the cross-links were between DNA and protein, since a heavily cross-linked sample regained most of its mobility upon treatment with proteinase K.[48] This additionally shows that the altered mobility patterns observed in lanes 5−7 were not a result of DNA decomposition, but rather of histone−DNA cross-linking. After formation of the adduct and digestion of the proteinaceous material, the plasmid remains mostly in supercoiled form but shows a slight increase in the amount of nicked form. Control samples omitting intercalator (lane 2), quencher (lane 3), or light (lane 4) all resembled the lane containing only plasmid; a small decrease in mobility is observed for the sample containing $Ru(phen)_2dppz^{2+}$ without quencher, indicating a small amount of cross-linking, presumably induced by singlet oxygen.[16,49]

We also followed the fate of the histone protein by gel electrophoresis. Figure 3 shows the results of a DNA−protein cross-linking experiment with a 20-mer oligonucleotide duplex, comprising 5′-GCAATCGTGCGTAGCAGAGC-3′ and its complementary strand. Irradiated samples of 20-mer duplex and histone, in the presence of intercalator and quencher, were analyzed by SDS-PAGE on a 10% polyacrylamide gel. Without irradiation, the histone runs primarily as a double band (lane 5). As the irradiation time increases, the intensity of the double band decreases, and other broad bands with decreased mobility appear (lanes 6−8). The control samples in lanes 1−4, irradiated for 4 min, are all very similar. These results confirm that both intercalator and quencher are required for the cross-linking reaction. Use of the single-stranded oligonucleotides also gave

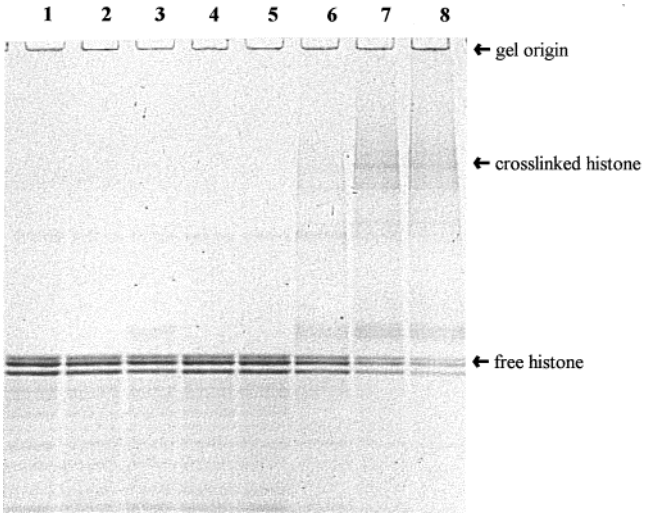

Figure 3. Denaturing polyacrylamide gel electrophoresis of histone following cross-linking to a DNA 20-mer duplex by the flash quench technique. (A) Samples shown are as follows: lane 1, histone irradiated for 240 s; lane 2, histone + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ irradiated for 240 s; lane 3, DNA + histone + $Ru(phen)_2dppz^{2+}$ irradiated for 240 s; lane 4, DNA + histone + $Co(NH_3)_5Cl^{2+}$ irradiated for 240 s: lane 5, DNA + histone + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ irradiated for 0 s; lane 6, DNA + histone + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ irradiated for 30 s; lane 7, DNA + histone + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ irradiated for 120 s; lane 8, DNA + histone + $Ru(phen)_2dppz^{2+}$ + $Co(NH_3)_5Cl^{2+}$ irradiated for 240 s. Conditions for irradiation: same as in Figure 1, but with 2.7 mW 442 nm light and a 20-mer duplex (25 μM) as the DNA.

rise to measurable, although diminished, levels of cross-linking. This is not unexpected, given that $Ru(phen)_2dppz^{2+}$ is also quenched efficiently in other less-ordered structures, such as micelles.[50]

Comparison of Assays Used To Detect the Cross-Links. In all of the assays, it was seen that increasing irradiation time increases the amount of DNA−protein cross-links when both intercalator and quencher are present with DNA and histone. When compared to the chloroform extraction assay, the filter binding assay appears to be less sensitive in detecting DNA−protein cross-links, as seen also by Mandel et al., who used these assays to detect DNA−histone cross-linking induced by UV light.[33a] The chloroform extraction and filter binding assays have the advantages of being quick and quantitative, but do not permit the direct observation of the cross-linked material. In contrast, the gel electrophoresis experiments allow one to follow the disappearance of free DNA or protein and its appearance as cross-linked material. During these experiments, it became apparent that the cross-linked material was reluctant to enter the gel matrix, possibly as a result of reduced solubility. Similar observations were made by Gebicki and Gebicki, who found that cross-linking experiments between plasmid DNA and histone protein failed to produce material detectable by agarose gel electrophoresis.[31]

Requirement of Guanine for Cross-Linking. To confirm that guanine bases are necessary for cross-linking, we examined the cross-linking as function of DNA composition. Figure 4**A** shows cross-linking profiles for poly(dA-dT), poly(dG-dC), and calf thymus DNA, using $Co(NH_3)_5Cl^{2+}$ as the quencher. As expected, the polymer lacking guanine bases undergoes only minimal cross-linking, whereas both poly(dG-dC) and CT DNA show substantial cross-linking. Clearly, cross-linking requires

(48) It is not surprising that a full restoration of mobility is not observed, since the protease will likely leave a peptide fragment on the DNA, owing to steric restraints.

(49) The metal-to-ligand charge-transfer excited states of $Ru(phen)_2dppz^{2+}$ and other ruthenium polypyridyl complexes, which have considerable triplet character, can damage guanine by a type II mechanism, i.e., production of singlet oxygen. The involvement of singlet oxygen is indicated by an enhancement of damage in the presence of D_2O. See refs 16 and 17, and: (a) Mei, H.-Y.; Barton, J. K. *J. Am. Chem. Soc.* **1986**, *108*, 7414. (b) Barton, J. K.; Goldberg, J. M.; Kumar, C. V. *J. Am. Chem. Soc.* **1986**, *108*, 2081. (c) Jenkins, Y. C. Ph.D. Thesis, California Institute of Technology, 1996. The yield for damage of guanine bases from $^1O_2$ via DNA-bound $Ru(phen)_2dppz^{2+}$ is very small, and thus observed only at much longer irradiation times than are used in the cross-linking experiments.

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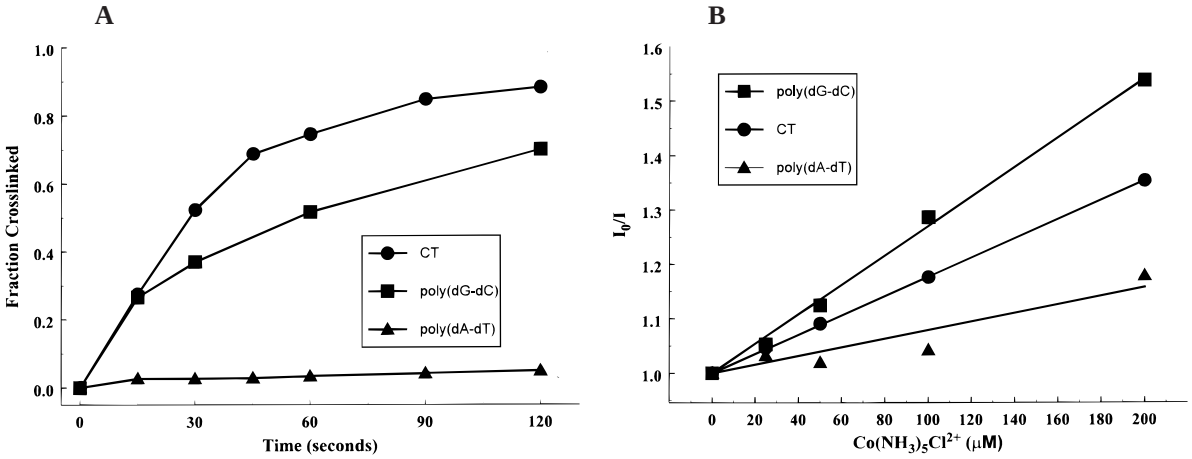

Figure 4. Dependence of cross-linking (A) and emission quenching (B) upon DNA composition. Shown are data for CT DNA(circles), poly(dG-dC) (squares) and poly(dA-dT) (triangles). The fraction of cross-linked DNA was determined by the chloroform extraction assay. The emission data are plotted as I_0/I vs [quencher], where I is the emission intensity and I_0 is the emission intensity in the absence of quencher. The lines are linear fits to the data, and the slopes represent the Stern−Volmer constants (K_{SV}). The excitation wavelength was 480 nm (2.4 mJ/pulse), and emission was monitored at 610 nm. Conditions: 10 μM $Ru(phen)_2dppz^{2+}$, 100 μM $Co(NH_3)_5Cl^{2+}$, 1 mM nucleotides DNA, and 250 μg/mL type III-S histone in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7.

Table 1: Quenching Parameters for DNA-bound $Ru(phen)_2dppz^{2+}$

DNA	quencher	histone	K_{sv} $(10^3\ M^{-1})^a$	k_q $(10^9\ M^{-1}\ s^{-1})^b$	τ_{avg} (ns)^c
CT	$Co(NH_3)_5Cl^{2+}$	+	1.8	7.8	231
CT	MV^{2+}	+	2.1	9.1	231
CT	$Ru(NH_3)_6^{3+}$	+	27	120	231
CT	$Co(NH_3)_5Cl^{2+}$	−	1.9	9.5	201
CT	MV^{2+}	−	4.7	23	201
CT	$Ru(NH_3)_6^{3+}$	−	29	140	201
poly(dA-dT)	$Co(NH_3)_5Cl^{2+}$	+	0.79	3.7	211
poly(dG-dC)	$Co(NH_3)_5Cl^{2+}$	+	2.7	11	238
poly(dG-dC)	MV^{2+}	+	4.3	18	238
poly(dG-dC)	$Ru(NH_3)_6^{3+}$	+	30	130	238

[a] Stern−Volmer constants were obtained from linear fits to I_0/I plots of the quenching data, where I = emission intensity and I_0 = emission intensity in the absence of quencher. [b] Quenching constants were obtained from $k_q = K_{SV}/\tau_{avg}$, where τ_{avg} is the average weighted lifetime of the unquenched emission. Individual emission lifetimes were determined from fits of the decay curves to $I(t) = I(t=0)\ [f \exp(-t/\tau_1) + (1-f)\exp(-t/\tau_2)]$. Uncertainties in lifetimes are estimated to be ~10%. [c] The lifetimes used to calculate τ_{avg} are: with CT DNA, τ_1 = 122 ns (83%) and τ_2 = 590 ns (17%); with CT DNA and histone, τ_1 = 108 ns (73%) and τ_2 = 562 ns (27%); with poly(dA-dT), τ_1 = 122 ns (84%) and τ_2 = 680 ns (16%); with poly(dG-dC), τ = 238 ns, as the unquenched emission was adequately described by a single-exponential decay.

guanine bases, as expected for a reaction involving guanine radical as an intermediate.

In a recent report,[51] the quenching of intercalated $Ru(phen)_2dppz^{2+}$ by $Ru(NH_3)_6^{3+}$ was shown to rely on composition, with more efficient quenching by the groove-bound acceptor seen in poly(dG-dC) than in poly(dA-dT). Hence, one might consider whether the observed difference in cross-linking is simply a result of the less efficient quenching in the AT polymer, since luminescence quenching titrations of $Ru(phen)_2dppz^{2+}$ with $Co(NH_3)_5Cl^{2+}$ show a similar trend with DNA composition (Figure 4B). However, this difference in quenching does not account for the lack of reactivity in the AT polymer, since the quenching constant in CT DNA, where considerable cross-linking is observed, is only ~2.5 times greater than for poly(dA-dT) (Table 1). Indeed, it is interesting that the CT DNA shows more cross-linking than poly(dG-dC), given

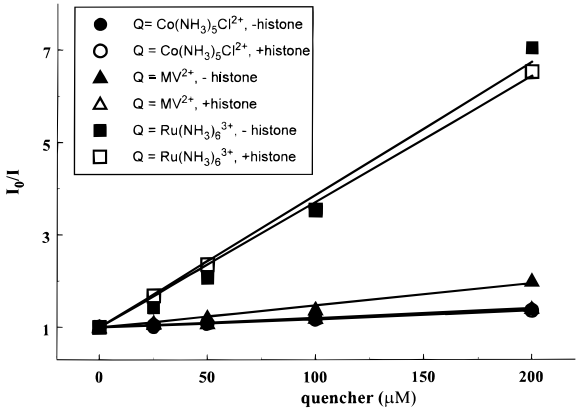

Figure 5. Emission quenching titrations of DNA-bound $Ru(phen)_2dppz^{2+}$ in the presence (open symbols) and absence (filled symbols) of histone. Shown are data for $Co(NH_3)_5Cl^{2+}$ (circles), MV^{2+} (triangles), and $Ru(NH_3)_6^{3+}$ (squares). The analysis of the emission data was the same as in Figure 4. Conditions: 10 μM $Ru(phen)_2dppz^{2+}$, 1 mM sonicated CT DNA, and either 0 or 250 μg/mL type III-S histone in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7.

that it is only 42% GC and has a smaller quenching constant. This observation may reflect the greater localization of the guanine radical in the natural DNA. If one considers the electron hole in terms of the polaron model of Schuster,[52] the energy of the polaron would certainly vary less in poly(dG-dC), which should facilitate hopping. In contrast, the sequence heterogeneity of the CT DNA should better localize the radical by providing low-energy sites such as GG or GGG, known to be especially susceptible to damage.[10,53] Given the slow nature of the cross-linking reaction (vide infra), this decrease in polaron-hopping efficiency would probably lead to more efficient cross-linking.

Quenching Titrations. To determine whether the quenchers function efficiently in the presence of histone on DNA, luminescence quenching titrations were carried out by means of time-resolved emission spectroscopy. Figure 5 shows titrations with $Co(NH_3)_5Cl^{2+}$, MV^{2+}, and $Ru(NH_3)_6^{3+}$ of $Ru(phen)_2$-

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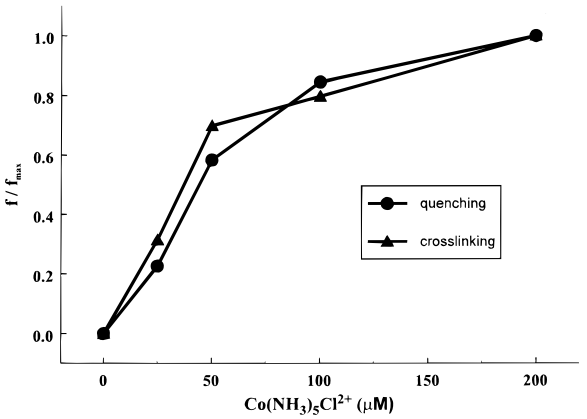

Figure 6. Correlation between cross-linking and quenching. The fraction cross-linked (triangles) and fraction quenched (circles) were determined as a function of quencher concentration, and are plotted as f/f_{max}, where the fraction of quenching/cross-linking has been normalized to the maximal fraction observed. The fraction cross-linked was determined by the chloroform extraction assay for samples irradiated for 30 s with 442 nm light (2.5 mW). Conditions: 10 μM Ru(phen)$_2$dppz^{2+}, 100 μM Co(NH$_3$)$_5$Cl^{2+}, 1 mM nucleotides sonicated CT DNA, and 250 μg/mL type III-S histone in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7.

dppz^{2+} bound to calf thymus DNA in the presence of histone. For all three quenchers, plots of I_0/I vs [quencher] are linear, and linear regression yields Stern−Volmer constants (K_{SV}) of 1.8×10^3 M^{-1}, 2.1×10^3 M^{-1}, and 2.7×10^4 M^{-1} for Co(NH$_3$)$_5$Cl^{2+}, MV^{2+}, and Ru(NH$_3$)$_6^{3+}$, respectively (Table 1). The decrease in emission intensity tracks well with the decrease in emission lifetimes (data not shown), indicating that the quenching process is dynamic, that is, that it occurs on the time scale of the measurement. The quenching efficiency decreases in the order Ru(NH$_3$)$_6^{3+}$ > MV^{2+} ≥ Co(NH$_3$)$_5$Cl^{2+}, in agreement with earlier results using oligonucleotide duplexes.[16] Converting the Stern−Volmer constants to quenching constants by dividing by the average emission lifetime, one obtains $k_q = 7.8 \times 10^9$ M^{-1} s^{-1}, 9.1×10^9 M^{-1} s^{-1}, and 1.2×10^{11} M^{-1} s^{-1} for Co(NH$_3$)$_5$Cl^{2+}, MV^{2+}, and Ru(NH$_3$)$_6^{3+}$, respectively. These values indicate efficient quenching of the DNA-bound metallointercalator.[54] When the titrations are carried out in absence of histone, the values of K_{SV} and k_q are somewhat higher, but comparable to those obtained in the presence of histone; the organic acceptor methyl viologen shows the largest difference. Thus, under these conditions, the histone does not deny access of the intercalator to the quencher.

Correlation between Quenching and Cross-Linking. To demonstrate that the quenching leads to cross-linking, the amount of quenching was compared with the amount of cross-linking at various concentrations of Co(NH$_3$)$_5$Cl^{2+}. The quenching and the cross-linking profiles (Figure 6) clearly show a similar dependence upon quencher concentration. Given that previous work has demonstrated that the amount of emission quenching also tracks well with the amount of guanine damage,[17] the correlation between quenching and cross-linking supports the hypothesis that oxidative damage to guanine, caused by the flash−quench technique, results in the formation of DNA−protein adducts.

Dependence of Cross-Linking Yield upon Quencher. Since the amount of permanent damage generated at guanine bases by the flash−quench technique has been shown to be modulated by the choice of quencher,[16] the dependence of cross-linking yield upon quencher identity was examined. Figure 7A shows the dependence of DNA−protein cross-linking upon quencher with calf thymus DNA. Using Co(NH$_3$)$_5$Cl^{2+} as the quencher, ~70% of the DNA is cross-linked to protein within 10 s and ~90% within 3 min of irradiation time. When MV^{2+} is used as the quencher, DNA−protein cross-links formed more slowly; only 40% is cross-linked at 10 s, and only 70% is cross-linked at 3 min. Samples with Ru(NH$_3$)$_6^{3+}$ as quencher gave significantly less cross-linking, with less than 4% of the DNA cross-linked to protein within 10 s and only ~20% within 3 min of irradiation time. Similar results were obtained with poly(dG-dC), as seen in Figure 7B. Thus, the yield of DNA−protein cross-linking decreases in the order Co(NH$_3$)$_5$Cl^{2+} > MV^{2+} > Ru(NH$_3$)$_6^{3+}$, mirroring earlier results correlating the amount of guanine damage as a function of quencher.

To understand this trend with quencher, one must consider the reactions that follow the quenching event. While the quenching efficiency decreases in the order Ru(NH$_3$)$_6^{3+}$ > MV^{2+} > Co(NH$_3$)$_5$Cl^{2+}, the opposite is observed for the cross-linking reaction. Thus, to identify the quencher that is most effective in cross-link generation, the amount of quenching is not the determining factor. Instead, the trend in cross-linking corresponds well with the lifetime of the guanine radical expected from the reactivity of the reduced quencher with the radical.[55] Although Ru(NH$_3$)$_6^{3+}$ quenches the most, it gives the least damage because of the efficient reaction between Ru(NH$_3$)$_6^{2+}$ and the guanine radical. MV^{2+} does not quench as well as Ru(NH$_3$)$_6^{3+}$, but more guanine damage is observed because reaction of MV^{1+} with guanine radical is less efficient; the methyl viologen cation reacts with O_2 to produce superoxide, O_2^-,[56] which reacts more slowly with the anionic DNA to regenerate the guanine base.[53b] Although it is the least efficient quencher, Co(NH$_3$)$_5$Cl^{2+} is the most efficient at producing damage at G. A sacrificial quencher, Co(NH$_3$)$_5$Cl^{2+} rapidly decomposes to Co(H$_2$O)$_6^{2+}$ upon reduction of the metal center to the labile d^7 configuration,[57] preventing further reaction.

Evidence for a Redox Quenching Mechanism. Using emission and transient absorption spectroscopies, the quenching of Ru(phen)$_2$dppz^{2+} by MV^{2+} was shown to occur via electron transfer. The appearance of the electron-transfer intermediate was monitored at 373 nm, a wavelength near the *Ru^{2+} − Ru^{2+} and Ru^{3+} − Ru^{2+} isosbestic points for the metal complex, so as to minimize any potential interference from excited state or Ru(III) intercalator. With poly(dG-dC), the quenching of *Ru(phen)$_2$dppz^{2+} by MV^{2+} results in a positive signal at 373 nm (Figure 8A). This 373 nm signal rises on the same time scale as the emission of *Ru(phen)$_2$dppz^{2+} decays, indicating that the intermediate is a product of the quenching.

The absorbance difference spectrum of the long-lived signal was generated (Figure 8B, circles), with the amplitude at various wavelengths determined from fits of the long-lived portion of the signal to an exponential function. The spectrum closely resembles that reported for MV^{1+}, with maxima at ~395 nm and ~610 nm,[58] confirming that MV^{2+} quenches *Ru(phen)$_2$dppz^{2+} by electron transfer. It is noteworthy that the difference spectrum is positive in the 400−500 nm region. The metal-to-

(54) Indeed the quenching here is more efficient than for oligonucleotide duplexes in the absence of histone. This can be attributed to the fact that the quenching process, which requires diffusion of the quencher to the metallointercalator along the DNA, is more efficient for longer strands of DNA.

(55) All of these quenchers typically function as 1-electron acceptors. Thus, the regeneration of guanine from the neutral guanine radical is likely to involve a 1-electron reduction, followed by protonation of the resulting guanine anion. Guanine has a pK_a of 9.5 (See ref 2b).

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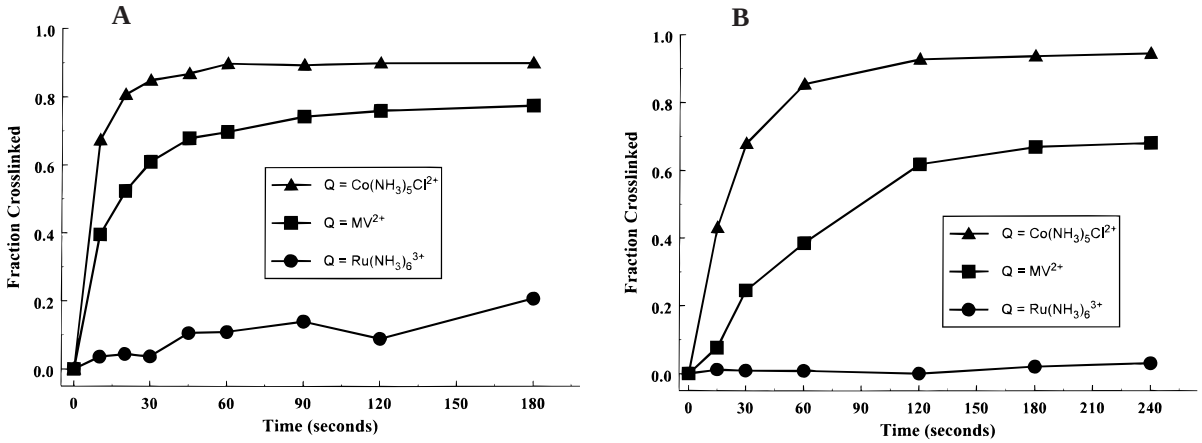

Figure 7. Dependence of DNA−protein cross-linking yield upon quencher, for calf thymus DNA (A) and poly(dG-dC) (B). Shown are data for samples employing $Co(NH_3)_5Cl^{2+}$ (triangles), MV^{2+} (squares) or $Ru(NH_3)_6^{3+}$ (circles) as quencher. Conditions: 10 μM $Ru(phen)_2dppz^{2+}$, 100 μM quencher, 1 mM DNA, and 250 μg/mL type III-S histone, in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7. Irradiation was carried out at 442 nm (∼5.5 mW), and DNA−protein cross-links were detected using the chloroform extraction assay.

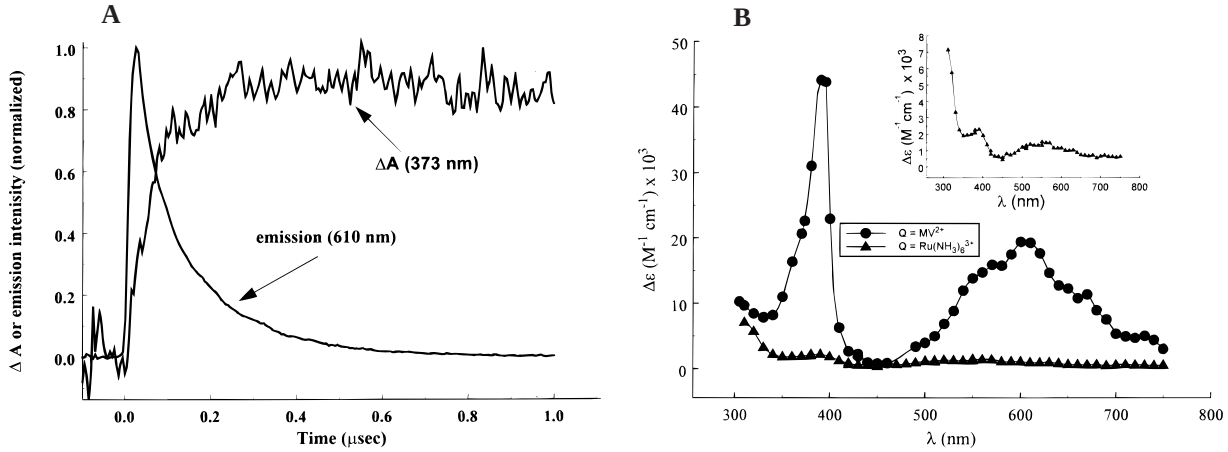

Figure 8. Detection by transient absorption spectroscopy of the intermediate formed upon quenching photoexcited $Ru(phen)_2dppz^{2+}$ in poly(dG-dC). (A) With methyl viologen as quencher, this plot shows the rise of the absorbance signal at 373 nm, overlaid with the emission decay signal for $*Ru(phen)_2dppz^{2+}$ at 610 nm. Both signals were normalized to the largest data value; the absorbance difference (ΔA) value for the 373 nm signal was ∼0.02 before normalization. (B) Spectra of long-lived transients produced when $Ru(phen)_2dppz^{2+}$ bound to poly(dG-dC) is quenched with methyl viologen (MV^{2+}, circles) or $Ru(NH_3)_6^{3+}$ (triangles). Inset: The spectrum of the transient produced by $Ru(NH_3)_6^{3+}$ quenching is shown on an expanded scale; this spectrum is adapted from ref 16 and closely resembles that of the neutral guanine radical (ref 4a). ΔA values were obtained from the raw data traces by fitting the data at $t > \tau(*Ru(phen)_2dppz^{2+})$ to an exponential function and extrapolating the fit back to time zero. For the spectrum of the guanine radical, ΔA values were then converted to $\Delta\epsilon$ values by assuming $\Delta\epsilon_{395} = 2.3 \times 10^3$ M^{-1} cm^{-1} (ref 4a). For the spectrum of the intermediate produced by MV^{2+} quenching, absorbance difference values were converted to $\Delta\epsilon$ values using $\Delta\epsilon_{395} = 4.4 \times 10^4$ M^{-1} cm^{-1} for the methyl viologen intermediate, as would be expected for an intermediate comprising MV^{1+} ($\Delta\epsilon_{395} = 4.2 \times 10^4$ $M^{-1}cm^{-1}$, ref 57) and $\Delta\epsilon_{395} = 2 \times 10^3$ M^{-1} cm^{-1} for the guanine radical. Conditions: 480 nm excitation (2 mJ/pulse), 20 μM $Ru(phen)_2dppz^{2+}$, 200 μM MV^{2+}, 2 mM nuc poly(dG-dC), in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7.

ligand charge transfer (MLCT) band of ruthenium polypyridyl complexes absorbs strongly in this range, so that the Ru^{3+} − Ru^{2+} spectrum shows a negative absorbance there. Thus, at ∼450 nm, where the MV^{1+} spectrum is nearly zero, and the MLCT absorbance of $Ru(phen)_2dppz^{2+}$ is near a maximum, one would expect a large negative signal if Ru(III) were present.[59] The fact that Ru(III) is not observed reflects the fact that, as observed with $Ru(NH_3)_6^{3+}$ as quencher, the oxidized metallointercalator is rapidly reduced by a guanine base. With $Ru(NH_3)_6^{3+}$ as quencher, the resulting intermediate in poly(dG-dC) has the spectrum of the neutral guanine radical (Figure 8B, triangles and Figure 8B, inset), with broad maxima near 390 and 550 nm.[16] It is difficult to obtain direct evidence for guanine radical with MV^{2+} as quencher, because the spectrum is dominated by the MV^{1+} chromophore. However, at ∼310 nm, where the MV^{1+} − MV^{2+} spectrum has an isosbestic point[58] and the guanine radical shows a positive absorbance,[16] the spectrum (Figure 8B) remains positive, consistent with the presence of the guanine radical as the other absorbing species.

Transient Absorption Measurements of the Guanine Radical Decay as a Function of Quencher. The observed dependence of cross-linking yield upon quencher thus provides information about the kinetics of the cross-linking reaction. Using transient absorption spectroscopy, we monitored the 373 nm signal for the guanine radical in poly(dG-dC),[16] produced by quenching photoexcited $Ru(phen)_2dppz^{2+}$ with $Ru(NH_3)_6^{3+}$ or $Co(NH_3)_5Cl^{2+}$. Upon photoexcitation, the rise of the 373 nm signal occurs concomitantly with the decay of $*Ru(phen)_2dppz^{2+}$, indicating that the quenching reaction is rate-limiting in the formation of the guanine radical in poly(dG-dC).[60] As shown

(59) With poly(dA-dT), the quenching of $Ru(phen)_2dppz^{2+}$ by MV^{2+} gives rise to a negative signal at 440 nm and positive signals at ∼395 nm and ∼610 nm, consistent with formation of a $Ru(phen)_2dppz^{3+}/MV^{1+}$ intermediate.

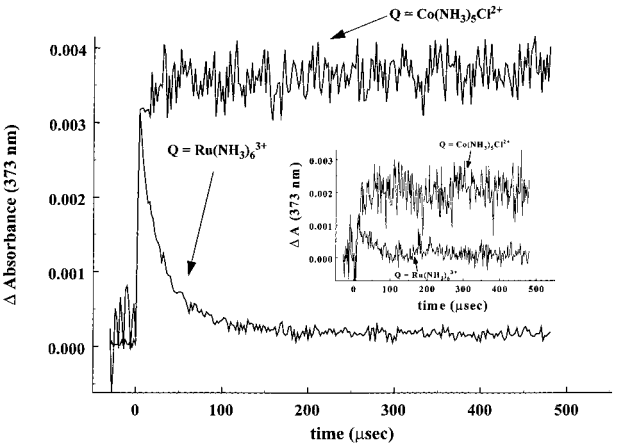

Figure 9. Transient absorption measurements of the guanine radical. Shown are long-lived transients at 373 nm formed upon quenching of photoexcited $Ru(phen)_2dppz^{2+}$ with $Ru(NH_3)_6^{3+}$ or $Co(NH_3)_5Cl^{2+}$ in poly(dG-dC). Inset: Same as above, except that histone was present at a concentration of 250 μg/mL. Samples containing histone transmitted much less probe light, likely owing to scattering, resulting in a decreased signal-to-noise ratio. Conditions: 480 nm excitation (2.6 mJ/pulse), 20 μM $Ru(phen)_2dppz^{2+}$, 2 mM nuc poly(dG-dC), and either 200 μM $Ru(NH_3)_6^{3+}$ or 500 μM $Co(NH_3)_5Cl^{2+}$, in a buffer of 10 mM sodium phosphate, 20 mM sodium chloride, pH 7.

in Figure 9, the guanine radical is efficiently reduced by $Ru(NH_3)_6^{2+}$, with only a small fraction of signal persisting beyond 100 μs. In contrast, when the sacrificial quencher $Co(NH_3)_5Cl^{2+}$ is used, the signal does not decay appreciably in the 500 μs time window, and persists well into the millisecond regime. Moreover, the presence of the histone does not appear to affect the kinetics of guanine radical reduction, as similar signals were observed (Figure 9, inset); this result is consistent with the fact that the histone does not influence the quenching reaction much either, and suggests that the cross-linking reaction is slow relative to the time scale of the measurement. Given that only a small amount of cross-linking is observed with $Ru(NH_3)_6^{3+}$ as quencher, the time required for cross-linking must be longer than that required for reaction of $Ru(NH_3)_6^{2+}$ with guanine radical, that is, ~100 μs. With the sacrificial quencher $Co(NH_3)_5Cl^{2+}$, the guanine radical has a much longer lifetime, and more cross-linking is observed. These results illustrate one clear advantage of the flash−quench approach, the production of a long-lived guanine radical for which the lifetime can be tuned. This permits the observation of both the permanent adducts resulting from guanine oxidation and of the guanine radical intermediate, making the flash−quench technique a powerful tool for determining the fates of the guanine radical.

Cross-Link Density. To estimate the density of cross-linked protein on the DNA, we carried out a flash−quench experiment, irradiating a sample of CT DNA, histone, $Ru(phen)_2dppz^{2+}$ and $Co(NH_3)_5Cl^{2+}$ with 442 nm light (6.2 mW). Image analysis of the protein bands from an SDS-PAGE experiment yielded the amount of free histone lost to cross-linking, while the chloroform extraction assay was used to obtain the fraction of cross-linked DNA. For a 2 min irradiation time, 63% of the free protein was lost, and 92% of the DNA was cross-linked. From these data and the average size of a sonicated calf thymus DNA molecule (~3000 bp), the average number of cross-links/DNA molecule was estimated to be ~68; this number corresponds to one protein per 44 base pairs of DNA. Thus, while the guanine radical can also form other products[3] or undergo repair by oxidizing the protein,[61] DNA−protein cross-linking appears to be a significant reaction pathway.

Decay Pathways of the Guanine Radical. The 1-electron oxidation of guanine leads initially to the guanine cation radical.[2b] In the nucleoside form, the guanine cation radical rapidly deprotonates in solution, even when frozen.[62] Because we observed only the neutral form in poly(dG-dC) by transient absorption spectroscopy,[16] and because the cross-linking reaction appears to be slow relative to deprotonation (vide supra), it is reasonable to suggest that cross-linking occurs primarily via the neutral radical rather than the cation radical. However, there is some evidence to suggest that the guanine cation radical may persist in DNA.[63] In a model system for lysine−guanine cross-linking, Morin and Cadet observed two different adducts upon photooxidation in aqueous solution, which they attributed to the two forms of the guanine radical.[39b] Thus, DNA−protein cross-linking via attack of the guanine cation radical cannot be ruled out.

Nature of the Cross-Link. While the proteinase K experiments clearly indicate that protein is the species cross-linked to DNA, the amino acids participating in the cross-linking reaction have not been determined. The work of Morin and Cadet[38,39] suggests that possibilities include Ser, Thr, Lys, and Arg, with Lys a particularly likely candidate since the histone is a lysine-rich protein. They have shown that 1-electron oxidized guanine is susceptible to attack at C8, a position accessible to agents binding in the major groove of DNA, as is the case for many DNA-binding proteins.[40] Using a 2′-deoxyguanosine model compound containing a 5′-amino group, they found that this amine nucleophile attacks C8 of the radical to form a cyclic nucleoside.[39a] This adduct is analogous to that which would be formed with amino side chains of proteins, such as Lys. Indeed, upon tethering a lysine residue from the 5′ position of 2-deoxyguanosine, they again observed cross-linking to C8 of the guanine base following type I photooxidation.[39b] Two adducts were characterized, one arising from the cation radical and the other from the neutral radical. Moreover, type I photooxidation of an acetylated 2′-deoxyguanosine leads to addition of methanol at C8, possibly via a guanine radical intermediate formed upon addition of O_2 to the neutral radical.[37] For both nucleophiles, further reactions involving H_2O lead to the final product. In the case of DNA−histone cross-linking described here, it is not clear whether the solvent accessibility of the modified guanine bases will be sufficient for such reactions to occur; thus, the final products could be different. Investigations of the structure of the lesions, and the effects of O_2 thereon, are underway.

(60) Since the quenchers have different quenching efficiencies, the rise time of the electron-transfer intermediate, i.e., guanine radical and reduced quencher, varies somewhat with the quencher. It is fastest with $Ru(NH_3)_6^{3+}$ as quencher and slower with methyl viologen (Figure 8A) and the cobalt complex. The rate constant for oxidation of G by $Ru(phen)_2dppz^{3+}$ is not known, but from measurement with $Ru(NH_3)_6^{3+}$ as quencher, a lower limit of 2×10^7 s^{-1} has been estimated (see ref 16).

(61) (a) Cullis, P. M.; Jones, G. D. D.; Symons, M. C. R.; Lea, J. S. *Nature* **1987**, *330*, 773. (b) Stemp, E. D. A.; Barton, J. K., submitted for publication. (c) Wagenknecht, H.-A.; Stemp, E. D. A.; Barton, J. K., *J. Am. Chem. Soc.* **2000**, *122*, 1. (d) Transfer of the electron hole from guanine to protein may produce reactive intermediates on the protein that could participate in cross-linking reactions. We are investigating this possibility.

(62) (a) Ravkin, B.; Herak, J. N.; Voit, K.; Hüttermann, J. *Radiat. Environ. Biophys.* **1987**, *26*, 1. (b) Hole, E. O.; Nelson, W. H.; Close, D. M.; Sagstuen, E. *J. Chem. Phys.* **1987**, *86*, 5218.

(63) Two of the competing pathways for decay of the guanine cation radical are deprotonation and hydration. Formation of 8-oxo-G, which proceeds via hydration of the cation radical, is greatly enhanced when the guanine is in DNA rather than in solution as a free nucleoside. Hydration apparently competes more favorably with deprotonation once the guanine base is part of the DNA polymer. See refs 8d, 13 and 37.

Recently, Hickerson et al. reported cross-linking of MutY to DNA via oxidation of 8-oxo-G with K_2IrCl_6.[24] The cross-link formed between the amino group of Lys 142 and C5 of the 8-oxo-G. In earlier experiments using the flash−quench technique, 8-oxo-G was detected as a product when $*Ru(phen)_2dppz^{2+}$ was oxidized with $Ru(NH_3)_6^{3+}$, and thus DNA−protein cross-linking via oxidation of 8-oxo-G could occur. However, reaction of protein with the guanine radical rather than an 8-oxo-G radical seems more likely, given that cross-linking is observed under irradiation conditions that produced single-hit conditions in gel electrophoresis experiments used to quantify guanine damage.[16,64]

Some other ruthenium(II) polypyridyl complexes actually participate in cross-linking reactions with DNA.[65] However, we do not anticipate a ruthenium-containing cross-link, since the metal complex does not possess easily displaced ligands,[65b−d] nor does it directly photooxidize the DNA,[65a] the oxidized intercalator returning to its stable Ru(II) ground state after oxidation of guanine. Indeed, because of the mobility of the guanine radical in DNA, the protein need not be near the binding site of the intercalator. If such a requirement did exist, then the quenching would likely be far less efficient because of competitive binding of protein and metal complex to the DNA. Thus, the chemistry involved in the flash−quench reaction, as well as the efficiency of quenching and cross-linking observed, is not consistent with a metal-containing cross-link.

Evidence That Cross-Linking Results from the Flash−Quench Experiment. Several observations support the notion that 1-electron oxidation of guanine via the flash−quench technique leads to DNA−protein cross-linking. First, the flash−quench technique has been previously shown to generate damage selectively at guanine bases in DNA,[16] and we have observed the guanine radical by transient absorption spectroscopy. The flash−quench approach also generates DNA−protein cross-links, as observed in assays monitoring both the DNA and the protein. Guanine is required for the reaction, as is the presence of both the intercalator, $Ru(phen)_2dppz^{2+}$, and the quencher. Moreover, the fact that neither the intercalator nor the quencher alone cause cross-linking indicates that neither direct photooxidation nor singlet oxygen sensitization occur to a significant extent. All of these results are consistent with cross-link formation via guanine oxidation by the flash−quench technique.

Implications and Conclusions

Here, we have demonstrated for the first time that the formation of electron holes on guanine bases causes DNA−protein cross-linking. Given that cellular DNA is intimately associated with proteins and incurs hundreds of oxidative hits each day,[1c,66] such adducts are likely to be a major form of DNA damage.[21] This damage appears to be particularly important in view of recent findings pertaining to the status of histone acetylation/deacetylation processes, which play a key role in transcriptional viability.[67] While cells do repair DNA−protein cross-links, there is a scarcity of knowledge regarding the structure of the lesions and their repair, in part because cross-linking methods typically generate a plethora of products. As a route to selective oxidation of guanine bases in DNA, the flash−quench technique should elucidate an understanding of these guanine-containing adducts at the molecular level.

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(64) Oxidation with MV^{2+} or $Co(NH_3)_5Cl^{2+}$ as quencher does not produce detectable amounts of 8-oxo-G. Perhaps 8-oxo-G is oxidized further with these quenchers to produce an 8-oxo-G radical, which could react with protein by a mechanism similar to that reported by Hickerson et al. (ref 24). Alternatively, the longer lifetime of the guanine radical in these cases may be responsible for the different distribution of guanine oxidation products.

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(66) Cadet, J.; D'Ham, C.; Douki, T.; Pouget, J.-P.; Ravanat, J.-L.; Sauviago, S. *Free Radical Res.* **1998**, *29*, 541.

(67) Workman, J. L.; Kingston, R. E. *Annu. Rev. Biochem.* **1998**, *67*, 545. (b) Wade, P. A.; Pruss, D.; Wolffe, A. P. *Trends Biochem. Sci.* **1997**, *22*, 128.