DNA−Protein Cross-Linking via Guanine Oxidation: Dependence upon Protein and Photosensitizer†

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ABSTRACT: DNA−protein cross-links form when guanine undergoes a 1-electron oxidation in a flash−quench experiment, and the importance of reactive oxygen species, protein, and photosensitizer is examined here. In these experiments, a strong oxidant produced by oxidative quenching of a DNA-bound photosensitizer generates an oxidized guanine base that reacts with protein to form the covalent adduct. These cross-links are cleaved by hot piperidine and are not the result of reactive oxygen species, since neither a hydroxyl radical scavenger (mannitol) nor oxygen affects the yield of DNA−histone cross-linking, as determined via a chloroform extraction assay. The cross-linking yield depends on protein, decreasing as histone > cytochrome *c* > bovine serum albumin. The yield does not depend on the cytochrome oxidation state, suggesting that reduction of the guanine radical by ferrocytochrome *c* does not compete effectively with cross-linking. The photosensitizer strongly influences the cross-linking yield, which decreases in the order $Ru(phen)_2dppz^{2+}$ [phen = 1,10-phenanthroline; dppz = dipyridophenazine] > $Ru(bpy)_3^{2+}$ [bpy = 2,2′-bipyridine] > acridine orange > ethidium, in accordance with measured oxidation potentials. A long-lived transient absorption signal for ethidium dication in poly(dG-dC) confirms that guanine oxidation is inefficient for this photosensitizer. From a polyacrylamide sequencing gel of a ^{32}P-labeled 40-mer, all of these photosensitizers are shown to damage guanines preferentially at the 5′ G of 5′-GG-3′ steps, consistent with a 1-electron oxidation. Additional examination of ethidium shows that it can generate cross-links between histone and plasmid DNA (pUC19) and that the yield depends on the quencher. Altogether, these results illustrate the versatility of the flash−quench technique as a way to generate physiologically relevant DNA−protein adducts via the oxidation of guanine and expand the scope of such cross-linking reactions to include proteins that may associate only transiently with DNA.

Oxidative damage to DNA is one significant outcome of oxidative stress and plays an important role in aging, chronic inflammatory diseases, neurodegenerative diseases, and cancer (*1*−*3*). Common forms of oxidative damage to DNA include oxidized bases such as 8-oxo-G[1] or thymine glycol (*4*). Another possible consequence of oxidative damage is the production of covalent adducts between DNA and protein, called DNA−protein cross-links (*5*).

As the DNA base with the lowest oxidation potential, guanine plays a special role because it is the most susceptible to oxidative damage (*6*−*8*). Moreover, the oxidation potential of guanine depends on the flanking sequence (*9*, *10*), and 5′-GG-3′ sites have been shown to be particularly vulnerable to damage (*11*−*15*). Guanine radicals are also mobile, and indeed, guanines can be damaged at a long distance from the site of initial oxidation (*11*, *12*, *16*−*20*). Currently, many groups are studying the ability of 5′-GG-3′ and 5′-GGG-3′ sequences (*21*−*23*) and low potential bases (*24*−*27*) to act as hole traps.

In recent years, several experimental approaches have been developed for selective oxidation of guanines in double-stranded DNA. Perhaps the most common method has been type I photooxidation (*28*), which has been used with anthraquinones (*18*), naphthalamides (*29*, *30*), rhodium complexes (*11*, *12*), and riboflavin (*31*). Other successful routes to guanine oxidation include photoionization by UV light (*32*−*34*), Norrish cleavage at DNA sugars (*19*, *20*), electrochemistry (*35*, *36*), and the flash−quench technique (*37*).

Initially used to study electron transfer in proteins (*38*), the flash−quench technique has proven to be a powerful tool for studies of guanine oxidation, since it allows the observation of guanine radical intermediates (*37*) as well as permanently damaged guanine products (*16*, *37*). Applied to DNA, the flash−quench technique produces a strong oxidant on the DNA by oxidative quenching of a photoex-

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[1] Abbreviations: phen, 1,10-phenanthroline; bpy, 2,2-bipyridine; dppz, dipyridophenazine; G, guanine; 8-oxo-G, 7,8-dihydro-8-oxoguanine; FAPy-G, 2,6-diamino-5-formamido-4-hydroxypyrimidine; SDS, sodium dodecyl sulfate; CT DNA, calf thymus DNA; UV, ultraviolet; MLCT, metal-to-ligand charge transfer; TBE, Tris/borate/ethylenediaminetetraacetic acid; BSA, bovine serum albumin; cyt *c*, cytochrome *c*; nuc, nucleotides; NaP_i, sodium phosphate; MV^{2+}, methyl viologen; AO^{1+}, acridine orange; Et^{1+}, ethidium; *X, photoexcited form of molecule X; PS, photosensitizer.

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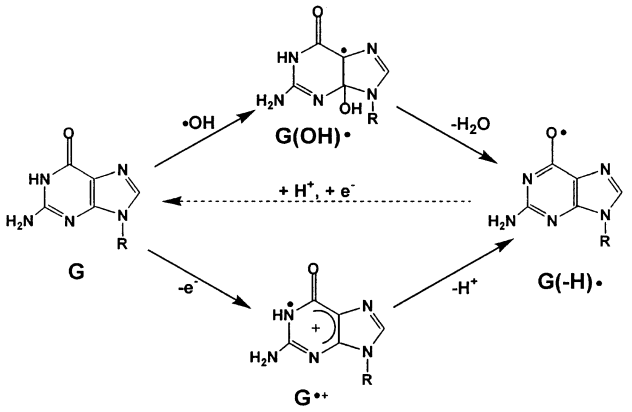
Scheme 1



cited intercalator. Dunn et al. first employed this approach in DNA, using excited ethidium quenched by methyl viologen to effect guanine-specific damage (*39*). With Ru(phen)$_2$dppz^{2+} (phen = 1,10-phenanthroline; dppz = dipyridophenazine) as intercalator and Ru(NH$_3$)$_6^{3+}$ as quencher, guanines in poly(dG-dC) were oxidized by flash−quench and shown via transient absorption spectroscopy to have the UV−visible spectrum of the neutral radical (*37*), as reported by Candeias and Steenken (*40*). The amount of irreversible guanine damage, as observed by gel electrophoresis, depends on the quencher, with higher yields observed with the quenchers that give longer lifetimes of guanine radical (*37*). Moreover, the permanent damage is localized in GG sequences when such hot spots are present (*16*, *37*) and can be initiated at distances of up to 200 Å from the intercalator (*12*). Recently, the flash−quench technique has also been employed to study protein to DNA electron transfer: specifically the reduction of guanine radical by ferrocytochrome *c* (*41*) and by aromatic amino acids in cationic tripeptides (*42*, *43*) and the DNA-binding protein, HhaI (*44*).

The flash−quench technique offers a way to remove an electron from the guanine base, presumably producing the cation radical, G•$^+$, en route to the neutral guanine radical, G(−H)• (*37*). As shown in Scheme 1, the neutral guanine radical is thought to be formed in two ways in vivo, either by (i) the 1-electron oxidation of guanine, followed by deprotonation of the cation radical, or by (ii) attack of the hydroxyl radical on guanine, followed by dehydration of the OH adduct, G(OH)• (*7*, *45*). Thus the flash−quench technique produces a physiologically relevant guanine radical for study.

Oxidation of G can lead to the formation of several permanent damage products, including covalent adducts. The guanine cation radical can be hydrated to form 8-oxo-G and FAPy-G, while the neutral form has been suggested as the precursor to oxazalone and imidazolone products (*31*, *45*, *46*); additional hydantoin products can also be formed from further oxidation of 8-oxo-G (*47*−*49*). Additionally, guanine radicals can undergo attack from nucleophiles to form covalent adducts. In well-defined model systems, Morin and Cadet have previously shown that 1-electron oxidation of 2′-deoxyguanosine leads to cross-links at C8 of guanine with either amino- or hydroxy-based nucleophiles (*50*−*52*); this work suggested that DNA−protein cross-links could be formed by reactions of oxidized guanine with Ser, Thr, and Lys. Oxidation of guanine damage products can also result in adduct formation, as Hickerson et al. have demonstrated that oxidation of 8-oxo-G in DNA by Ir(IV) leads to specific cross-linking between C5 of 8-oxo-G and lysine 142 of the DNA repair protein MutY (*53*). Last, guanine oxidation can lead to cross-links that do not contain guanine; oxidation of Tyr in Lys-Tyr-Lys peptides by guanine radical results in a tyrosine−thymine cross-link (*43*).

DNA−protein cross-linking represents a major form of DNA damage (*5*) and is emerging as a biomarker for carcinogenesis. A large number of agents can produce DNA−protein adducts, including cisplatin (*5*), Ir(IV) (*53*), Ni(II) (*54*), Cr(VI) (*55*), phenanthrolinecopper(II) (*56*), Fe(II) (*57*), Fe(III) bleomycin (*56*), aldehydes (*58*, *59*), hydroquinone (*60*), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (*61*), HOCl (*62*), peroxidized proteins (*63*), magnetic fields (*64*), ultraviolet light (*65*, *66*), X-rays (*67*), γ-rays (*68*), and visible light with $^1O_2$ sensitizers (*69*). Given that many of these agents have multiple targets, the molecular details of DNA−protein cross-linking remain poorly understood.

Since the flash−quench technique allows for the selective oxidation of guanine bases (*37*), we recently introduced this methodology as an effective means for inducing the formation of a single class of DNA−protein cross-links (*70*), those formed via 1-electron oxidation of the guanine base. Using Ru(phen)$_2$dppz^{2+} as the intercalator, we found that the cross-linking requires guanine bases and that the amount of cross-linking correlates with the amount of quenching, as expected for a process involving guanine radicals formed by the quenching event. As with guanine oxidation, the yield of cross-linking was strongly influenced by the identity of the quencher, decreasing in the order Co(NH$_3$)$_5$Cl^{2+} > methyl viologen > Ru(NH$_3$)$_6^{3+}$. Transient absorption measurements showed that a long-lived guanine radical is associated with efficient cross-linking (*70*).

Here, we have examined further the nature of the cross-linking reaction and have determined the effects of protein and photosensitizer on DNA−protein cross-linking via the flash−quench technique. Neither a hydroxyl radical scavenger nor the presence of oxygen affects the yield of cross-linking, which is consistent with our hypothesis that cross-linking results from guanine radicals rather than reactive oxygen species. Comparing the proteins histone, cytochrome *c* (cyt *c*), and bovine serum albumin (BSA), we find that cross-linking decreases in the order histone > cyt *c* > BSA. Looking more closely at cyt *c*, we find that the oxidation state of the heme does not significantly influence the yield of cross-linking. For photosensitizer, we examined other lumiphores more common than Ru(phen)$_2$dppz^{2+}, such as the organic intercalators ethidium and acridine orange, and the metal complex Ru(bpy)$_3^{2+}$. All of these compounds generated detectable cross-linking via the flash−quench technique, with the cross-link yields decreasing in the order Ru(phen)$_2$dppz^{2+} > Ru(bpy)$_3^{2+}$ > acridine orange > ethidium. We examine further the reaction with ethidium and show that the yield of cross-linking depends on the quencher, as shown for Ru(phen)$_2$dppz^{2+}. These results expand the scope and applicability of DNA−protein cross-linking reactions caused by guanine oxidation and suggest that such reactions can occur even with nonphysiological protein partners that associate only transiently with the DNA.

EXPERIMENTAL PROCEDURES

Materials. Sodium phosphate, sodium chloride, and sodium hydroxide were obtained from Mallinckrodt. 2-Mercaptoethanol, 24:1 chloroform−isoamyl alcohol, acridine orange (99%), ethidium bromide, hexaammineruthenium(III) chloride, chloropentaamminecobalt(III) chloride, pentaammineruthenium(III) chloride, potassium ferricyanide, and methyl viologen were obtained from Aldrich. Ultrapure agarose was obtained from Gibco BRL. Urea was purchased from ICN. Bovine serum albumin and electrophoresis grade sodium dodecyl sulfate (SDS) were purchased from Bio-Rad. $[Ru(phen)_2dppz]Cl_2$ was prepared as described previously (*71*). Horse heart cytochrome *c* and histone 3-S from calf thymus were obtained from Sigma. Unless otherwise stated, cytochrome *c* was used as received; if a specific oxidation state was desired, a concentrated solution of the cytochrome was either reduced with 2-mercaptoethanol or oxidized with $K_3Fe(CN)_6$ and the excess redox reagent removed via gel filtration chromatography (Sephadex G-25; Amersham Pharmacia) prior to use. pUC19 plasmid was purchased from New England Biolabs. Proteinase K, sonicated calf thymus DNA (CT DNA), and poly(dG-dC) 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 and ∼750 bp for poly(dG-dC). Prior to use, solutions of DNA polymers were exchanged into a buffer of 10 mM sodium phosphate (NaP_i) and 20 mM NaCl (pH 7) via multiple rounds of ultrafiltration using Centricon 30 microconcentrators (Amicon). Stock solutions were prepared by utilizing the following extinction coefficients: $\epsilon_{276} = 0.46$ mM^{-1} cm^{-1} for $Ru(NH_3)_6^{3+}$ (*72*), $\epsilon_{440} = 21$ mM^{-1} cm^{-1} for $Ru(phen)_2dppz^{2+}$ (*73*), $\epsilon_{452} = 14.5$ mM^{-1} cm^{-1} for $Ru(bpy)_3^{2+}$ (*74*), $\epsilon_{490} = 69$ mM^{-1} cm^{-1} for acridine orange (*75*), $\epsilon_{480} = 5.45$ mM^{-1} cm^{-1} for ethidium (*76*), $\epsilon_{257} = 20.7$ mM^{-1} cm^{-1} for methyl viologen (*77*), $\epsilon_{410} = 106$ mM^{-1} cm^{-1} for cytochrome *c* (*78*), $\epsilon_{260} = 6.6$ mM^{-1} cm^{-1} for calf thymus and pUC19 DNA, and $\epsilon_{254} = 8.4$ mM^{-1} cm^{-1} for poly(dG-dC); extinction coefficients for DNA are from the manufacturers, and concentrations of DNA are given in nucleotides (nuc). $Co(NH_3)_5Cl_3$, histone, and BSA were quantified by weight. Since the cobalt complex slowly decomposes at room temperature, solutions were frozen as aliquots, and a fresh aliquot was used for each experiment.

Sample Preparation. Samples (50−100 μL) containing 10 μM photosensitizer [$Ru(phen)_2dppz^{2+}$, $Ru(bpy)_3^{2+}$, acridine orange, or ethidium], 100 μM quencher [$Co(NH_3)_5Cl^{2+}$, $Ru(NH_3)_6^{3+}$, or methyl viologen], sonicated CT DNA (1 mM nucleotides), and 250 μg/mL protein (histone, cytochrome *c* or BSA) were prepared in a pH 7 buffer of 10 mM NaP_i and 20 mM NaCl. DNA was added last, under conditions of reduced light. Microcentrifuge tubes containing sample were placed with caps open toward the light source. Samples were then irradiated at 442 or 488 nm with a 1000 W Xe lamp or a 1000 W HgXe lamp, with powers of 0.8−5 mW. After irradiation, the samples were kept from exposure to light until the assay for cross-link detection (chloroform extraction or gel shift) was performed.

Preparation of Deoxygenated Samples. Samples were prepared under the conditions given above, except that the samples were contained in small glass vials covered with rubber septa; here the light source needed to pass through the curved glass wall to contact the sample, resulting in a less efficient irradiation. The air in the solutions was exchanged with nitrogen or argon by gently bubbling the gases through the corresponding solutions, while control samples were bubbled through with air.

Piperidine Treatment. Chemically modified bases can be liberated by treatment with hot alkali (*4*). Flash−quench samples (90 μL) were prepared and irradiated as above. Following irradiation, piperidine (10 μL), or buffer (10 μL) for control samples, was added to the samples. The samples containing piperidine, as well as one set of control samples containing no piperidine, were then heated at 90 °C for 30 min.

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% SDS and 1 M NaCl. Each solution was vortexed vigorously for 15 s, mixed with two volumes of chloroform−isoamyl 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 using the equation:

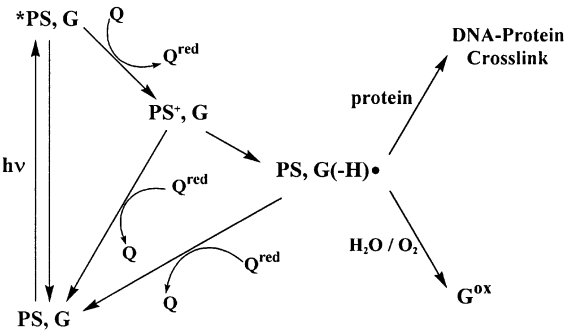
$$\text{fraction cross-linked} = [A_{260}(0) - A_{260}(t)]/A_{260}(0) \quad (1)$$

where $A_{260}(0)$ is the 260 nm absorbance of the unirradiated control sample and $A_{260}(t)$ is the 260 nm absorbance of a sample irradiated for time *t*.

Gel Shift Assay. DNA−protein cross-linking can also be detected using a gel shift assay (*79*). These experiments employed pUC19 DNA (1 mM nuc), ethidium (10 μM) as intercalator, $Co(NH_3)_5Cl^{2+}$ (100 μM) as quencher, and histone (250 μg/mL) as the protein. Samples (10 μL) were irradiated for 0−40 min and then diluted 1:1 with 10% SDS. When digestion of the cross-link was desired, proteinase K was added to a final concentration of 100 μg/mL, and the sample was incubated at 37 °C for 1 h. Loading buffer, which contained bromophenol blue and xylene cyanol as tracking dyes in 5× TBE buffer, was then added and diluted to a final concentration of 1× TBE (89 mM Tris−borate, 2 mM EDTA, pH 8.3). Samples containing ∼1.5 μg of DNA per well were run on a 0.8% agarose gel in a running buffer of 1× TBE at ∼80 V for 2.5 h. The gel was stained for 1.5 h using a 5 μM ethidium solution in 1× TBE and then destained for ∼2 h in 1× TBE containing 1 mM $MgSO_4$.

Photophysical Measurements. Time-resolved luminescence and absorption measurements utilized the 470−490 nm output (1−5 mJ/pulse, pulse width ∼8 ns) of a YAG-OPO laser in the Beckman Institute Laser Resource Center (*44*). Emission of $Ru(phen)_2dppz^{2+}$ was monitored at 610 nm and the emission intensity was obtained by integrating under the luminescence decay curve. Lifetimes were obtained by fitting the decay curves to exponential functions using in-house software. Emission quenching experiments comparing the different photosensitizers and different quenchers were performed on a steady-state spectrofluorometer (AB2; National Instruments).

Scheme 2



Cyclic Voltammetry. The oxidation potentials of the photosensitizers were estimated by cyclic voltammetry using a CV-50W instrument (Bioanalytical Systems). For these measurements, the working electrode was a glassy carbon electrode, and the reference electrode was Ag/AgCl. Each photosensitizer was converted into its hexafluorophosphate (PF_6^-) salt and dissolved in dry acetonitrile prior to the measurements. For $Ru(phen)_2dppz^{2+}$ and $Ru(bpy)_3^{2+}$, the oxidation process is reversible, and for acridine orange, the oxidation is quasi-reversible. Because the oxidation of ethidium is irreversible, only an estimate of the upper limit of the oxidation potential is reported.

Preparation of Oligonucleotides. Oligonucleotide strands were synthesized on an ABI DNA/RNA synthesizer (Applied Biosystems). Following initial purification by reversed-phase HPLC in the trityl-on form, the strands were then purified further by reversed-phase HPLC in the trityl-off form. The guanine-rich strand was labeled at the 5′ end with [γ-^{32}P]-ATP by polynucleotide kinase using standard protocols (*80*) and purified on a preparative polyacrylamide gel. The oligonucleotide duplex was formed by heating a solution containing the strands in a 1:1 ratio to 90 °C, followed by slow cooling over 90 min to room temperature.

RESULTS

Description of the Flash−Quench Technique. The flash−quench technique offers a simple way to produce the guanine radical by using visible light to create a strong ground state oxidant bound to the DNA (*37*). The potent oxidant, created in situ by oxidative quenching of an excited photosensitizer bound to DNA, removes an electron from guanine to make the guanine radical. In this method (Scheme 2), a ground state photosensitizer, PS, is excited with visible light to form the excited state *PS, which can then transfer an electron to quencher Q. The oxidant thus formed, PS^+, can either undergo back-electron transfer with reduced quencher Q^{red} or oxidize guanine, G. The resulting guanine radical cation is acidic (pK_a 3.9) and rapidly deprotonates to form the neutral radical G(−H)• (*40*). This radical can either be repaired by electron transfer from Q^{red} or react further to yield permanent damage in the form of G^{ox} or a DNA−protein cross-link.

Here, Q was typically $Co(NH_3)_5Cl^{2+}$, but methyl viologen (MV^{2+}) and $Ru(NH_3)_6^{3+}$ were also used for comparison purposes; the cobalt complex is the quencher of choice because its reduced form decomposes and thus does not reduce either PS^+ or G(−H)•. The photosensitizer PS represents $Ru(phen)_2dppz^{2+}$, $Ru(bpy)_3^{2+}$, acridine orange (AO^{1+}), or ethidium (Et^{1+}). For $Ru(phen)_2dppz^{2+}$ and $Ru(bpy)_3^{2+}$, the E^0 (Ru^{3+}/Ru^{2+}) values were measured to be +1.66 and +1.56 V, respectively (Table 1). The AO^{2+}/AO^{1+} couple was estimated to be +1.47 V from cyclic voltammetry measurements, and the E^0 for the Et^{2+}/Et^{1+} couple has an upper limit of +1.43 V (Table 1). Given that the reduction potential is +1.29 V for the neutral guanine radical (*8*) and that oxidized ethidium has been shown to cause guanine damage (*39*), all of the oxidized photosensitizers are presumed to be capable of guanine oxidation.

Table 1: Oxidation Potentials

compound[a]	E^0 (V)[b]
$Ru(phen)_2dppz^{2+}$	1.66
$Ru(bpy)_3^{2+}$	1.56
acridine orange	1.47[c]
ethidium	<1.43[d]
guanine	1.29[e]

[a] Measured in acetonitrile with photosensitizer present as PF_6^- salt. [b] Values vs NHE obtained by addition of 0.22 V to E^0 values obtained with Ag/AgCl electrode. [c] Quasi-reversible. [d] Irreversible; value given is upper limit estimated from the oxidation wave. [e] Obtained in water at pH 7 (*8*).

Reactive Oxygen Species. Previously, we demonstrated that quenching of photoexcited, DNA-bound $Ru(phen)_2dppz^{2+}$ leads to the formation of cross-links between DNA and histone (*70*). Cross-link formation was shown to require the presence of guanine, consistent with a process involving guanine radical as an intermediate. To demonstrate that reactive oxygen species (ROS) do not contribute to the cross-linking, the cross-linking reaction was examined under conditions disfavoring ROS formation.

To determine the significance of hydroxyl radicals in the cross-linking reaction, flash−quench samples were irradiated in the presence of mannitol, a hydroxyl radical scavenger (*81*). The samples contained mannitol, $Ru(phen)_2dppz^{2+}$, $Co(NH_3)_5Cl^{2+}$, sonicated CT DNA, and histone 3-S, and cross-links were detected by the chloroform extraction assay. As shown in Figure 1A, the fraction cross-linked was found to increase with increasing irradiation times, reaching a maximum of 0.8 at 2 min irradiation time both in the presence and in the absence of mannitol. As the fraction cross-linked does not appear to be influenced by mannitol, it is concluded that hydroxyl radicals do not participate in the cross-linking reaction.

Oxygen could contribute to cross-linking in a number of ways, since it can react with guanine radical intermediates (*45*, *46*) or be converted to singlet oxygen to carry out a type II photooxidation (*28*). To determine whether oxygen is required for the cross-linking reaction, flash−quench samples were irradiated under both aerobic and anaerobic conditions. The fraction cross-linked, as detected by the chloroform extraction assay, was found to increase with increasing irradiation times, plateauing at ∼0.3 at 200 s (Figure 1B). More importantly, the cross-linking yield for deaerated samples closely corresponds with the yield derived from aerated samples. The data indicate that oxygen is not required for the cross-linking reaction.

It is apparent that the absolute amount of cross-linking is significantly diminished in the experiments examining the effect of oxygen. These samples were irradiated through the conical portion of a sealed glass vial, so certainly the

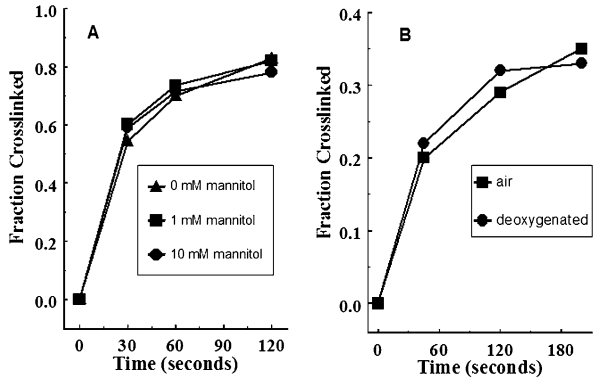


FIGURE 1: Effects of hydroxyl radical scavenger and deoxygenation on DNA−protein cross-linking via the flash−quench technique. (A) DNA−protein cross-linking detected by the chloroform extraction assay under conditions of 0 mM (triangles), 1 mM (squares), and 10 mM (circles) mannitol. (B) DNA−protein cross-linking detected by the chloroform extraction assay for aerated (squares) and deoxygenated (circles) samples. For samples in (B), the irradiation conditions were altered somewhat, leading to less efficient irradiation (see text). Conditions: 10 μM $Ru(phen)_2dppz^{2+}$, 100 μM $Co(NH_3)_5Cl^{2+}$, sonicated CT DNA (1 mM nuc), and 250 μg/mL histone 3-S in a pH 7 buffer of 10 mM NaP_i and 20 mM NaCl. 100 μL samples were irradiated at 442 nm (4 mW) with a 1000 W Xe lamp.

irradiation would be less efficient than for light contacting the solution directly, as for the samples in Figure 1A. However, if efficiency (e.g., kinetics) were the only factor, one might expect that the same yield of product should be obtained at longer irradiation times, which is not the case in Figure 1B. Perhaps the path of the excitation beam was perturbed enough upon passing through the curved glass that some portion of the sample volume was not irradiated or that the spectral distribution of the source was altered and caused photodecomposition of the cobalt quencher. Alternatively, some of the histone may have adsorbed onto the glass during the bubbling of the deaeration process, effectively decreasing the amount of protein available for cross-linking.

Alkali Lability of Cross-Links. It is well established that many types of base modifications render DNA susceptible to strand cleavage upon treatment with hot alkali (*4*). 8-Oxo-G is only sluggishly liberated, but other guanine lesions are cleaved more readily. With these observations in mind, we sought to determine whether treatment with hot piperidine cleaves cross-links between DNA and histone. Flash−quench samples containing $Ru(phen)_2dppz^{2+}$, $Co(NH_3)_5Cl^{2+}$, sonicated CT DNA, and histone were irradiated at 442 nm and then treated with hot piperidine prior to detection of cross-links by the chloroform extraction assay. The fraction cross-linked was found to increase with increasing irradiation time, plateauing at 0.8 for samples not treated with piperidine (Figure 2). Samples treated with piperidine showed no evidence of cross-linking. The data show that there is a slight decrease in the fraction cross-linked when the samples are heated, but the majority of the cleavage occurs as a result of the addition of piperidine; in contrast, samples heated at 90 °C for several hours do show a substantial decrease in cross-linking (data not shown). In summary, the data indicate that piperidine cleaves DNA−protein cross-links formed by guanine oxidation and that thermal cleavage of these cross-links is possible at much longer times.

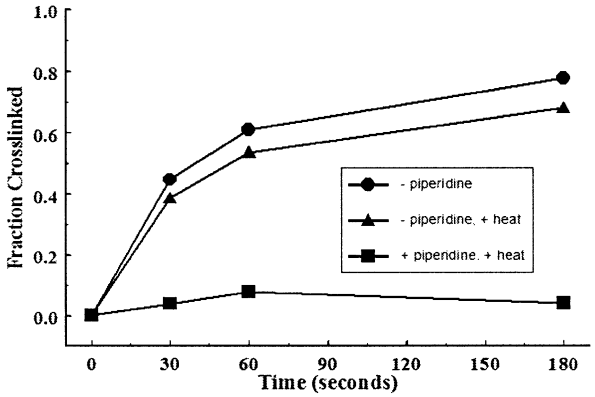


FIGURE 2: Sensitivity of DNA−protein cross-links to treatment with hot alkali. Shown are data for samples that were untreated (circles), treated with hot piperidine (squares), and treated with heat only (triangles). Irradiated samples treated with piperidine were brought to a concentration of 1 M piperidine and then heated at 90 °C for 30 min. 100 μL samples were irradiated with the 442 nm output (3 mW) of a 1000 W Xe lamp. Other conditions were as in Figure 1.

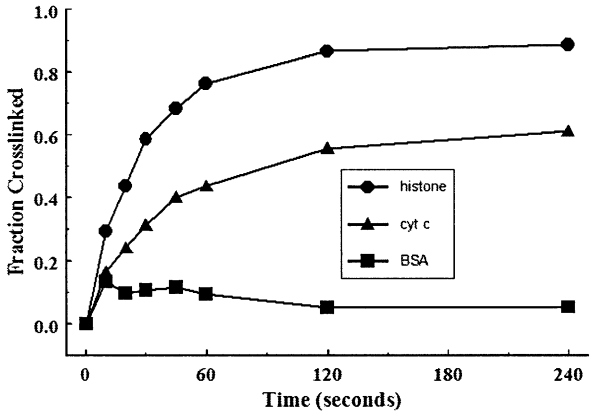


FIGURE 3: Dependence of DNA−protein cross-linking upon protein. Shown are data for histone (circles), cytochrome *c* (triangles), and BSA (squares). Cross-linking was detected by the chloroform extraction assay. All samples contained $Ru(phen)_2dppz^{2+}$ (10 μM), $Co(NH_3)_5Cl^{2+}$ (100 μM), CT DNA (1 mM nuc), and protein (250 μg/mL). Irradiation was carried out at 442 nm (4 mW) with a 1000 W Xe lamp.

Dependence of Cross-Linking Yield upon Protein. To gauge the range of proteins that might participate in cross-linking reactions with guanine radical, cytochrome *c* and bovine serum albumin were compared to histone 3-S, our benchmark protein. Histones are basic, lysine-rich, and physiological DNA-binding partners (*82*). Cytochrome *c*, though not a DNA-binding protein, is also a basic and lysine-rich protein (*83*) that interacts electrostatically with DNA. In contrast, bovine serum albumin is an acidic, cysteine-rich protein with no affinity for DNA. Figure 3 shows the cross-linking yields of these three different proteins with CT DNA, following quenching of $*Ru(phen)_2dppz^{2+}$ by $Co(NH_3)_5Cl^{2+}$. For the three samples that contained all four reactants, the fraction of DNA cross-linked to protein increases as irradiation time increases, leveling off at ~0.9 for histone, ~0.6 for cyt *c*, and ~0.1 for BSA, after 4 min of irradiation at 442 nm. Several other commercially available histones were also examined, and all of these showed substantial amounts of cross-linking (data not shown). Samples lacking either photosensitizer or quencher showed no significant cross-linking.

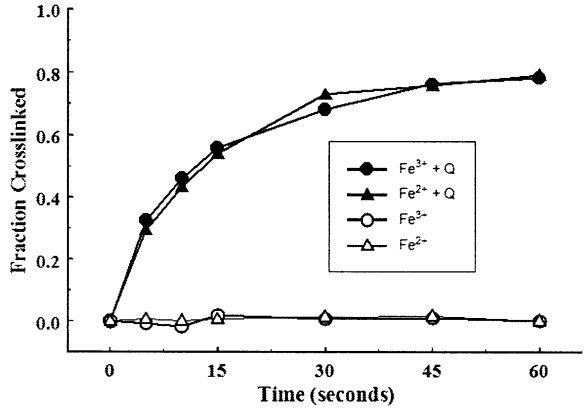


FIGURE 4: Dependence of DNA−cytochrome *c* cross-linking on heme oxidation state. Shown are data for Fe^{3+} cyt *c* (filled circles) and Fe^{2+} cyt *c* (filled triangles), along with control samples (corresponding open symbols) from which the quencher was omitted. Conditions: same as in Figure 1, except that poly(dG-dC) was used as the DNA.

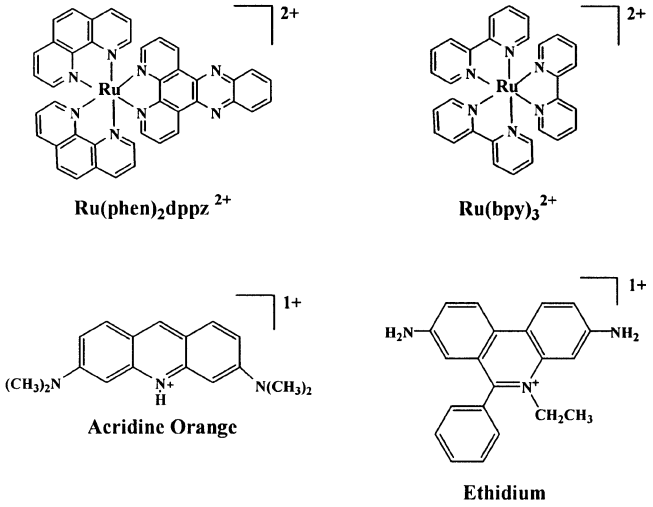


FIGURE 5: Structures of photosensitizers used in this study.

One possible reason for the differences in cross-linking by the three proteins is that the quenching efficiency is influenced by the identity of the protein. Thus, the quenching of $Ru(phen)_2dppz^{2+}$ emission by $Co(NH_3)_5Cl^{2+}$ was examined in the presence of each protein. As seen in Table 2, the amount of quenching of $Ru(phen)_2dppz^{2+}$ by $Co(NH_3)_5Cl^{2+}$, though similar for all three proteins, is actually highest with BSA, followed by histone and cyt *c*. Similar quenching results are obtained for $Ru(NH_3)_5Cl^{2+}$ (data not shown), a better electron acceptor which should interact with the DNA in a manner similar to that of $Co(NH_3)_5Cl^{2+}$. Thus, a difference in quenching efficiencies is not responsible for the different cross-linking yields.

Dependence of Cross-Linking on the Cyt c Redox State. In previous studies, ferrocytochrome *c* (Fe^{2+} cyt *c*) was found to be an efficient reductant of guanine radicals in DNA (*41*). Since DNA−protein cross-links generated by the flash−quench technique involve a guanine radical intermediate (*37*, *70*), it is logical to ask whether the redox state of the cytochrome influences the yield of DNA−cytochrome cross-linking. Figure 4 shows DNA−protein cross-linking detected by the chloroform extraction assay for samples containing $Ru(phen)_2dppz^{2+}$, $Co(NH_3)_5Cl^{2+}$, cytochrome *c*, and poly(dG-dC). Here, a fully GC polymer was used to ensure that all of the Ru(III) species are rapidly reduced, yielding guanine radicals (*37*). For the flash−quench samples containing all four reactants, the amount of cross-links increases smoothly as the irradiation time increases, leveling off at ~80% at longer times. Control samples in which the quencher was omitted gave little cross-linking. No significant difference in cross-linking between Fe^{3+} and Fe^{2+} cytochrome *c* was observed, signifying that the reduction of the guanine radical intermediate does not compete efficiently with cross-linking. Similar results were seen with CT DNA, which is only 42% GC (data not shown).

Given that Fe^{3+} cytochrome *c* can also quench excited $Ru(phen)_2dppz^{2+}$ (*41*), it was surprising that no cross-linking was observed in the absence of $Co(NH_3)_5Cl^{2+}$ (Figure 4). There are two possible scenarios to explain why there is no cross-linking without $Co(NH_3)_5Cl^{2+}$: (i) Fe^{3+} cytochrome *c* does not quench under these conditions, or (ii) if Fe^{3+} cytochrome *c* does quench, then the reduction of the guanine radical by DNA-bound Fe^{2+} cytochrome *c* is very fast. From emission measurements, it was found that Fe^{3+} cytochrome *c* does quench excited $Ru(phen)_2dppz^{2+}$ under these conditions, ruling out scenario i. Also, the Fe^{2+} cytochrome *c* was monitored at 550 nm by transient absorption spectroscopy, and the kinetics of the decay are very similar to those obtained earlier at a lower ionic strength. Nonetheless, as much as 60% of the excited $Ru(phen)_2dppz^{2+}$ quenched by Fe^{3+} cytochrome *c* could be undergoing a rapid back-electron transfer (*41*) or the resulting Fe^{2+} cyt *c* could cause rapid reduction of a nearby guanine radical, without yielding a long-lived guanine radical for cross-linking.

Dependence on Photosensitizer. In theory, DNA−protein cross-linking via flash−quench should be possible for any photosensitizer that binds to DNA and forms a strong oxidant upon quenching of its excited state. Therefore, we sought to expand the breadth of the flash−quench reaction by determining if other photosensitizers can induce cross-linking via this method. In addition to $Ru(phen)_2dppz^{2+}$, the benchmark for comparison, this study included $Ru(bpy)_3^{2+}$, ethidium, and acridine orange (Figure 5). These common photosensitizers vary in molecular structure, DNA-binding mode, lifetime and nature of the excited state, and propensity for singlet oxygen sensitization.

$Ru(phen)_2dppz^{2+}$. $Ru(phen)_2dppz^{2+}$ is a metallointercalator capable of selectively oxidizing guanine in the flash−quench reaction (*37*). A metal-to-ligand charge transfer (MLCT) occurs upon the excitation of the molecule with visible light, and this predominantly triplet excited state decays biexponentially for intercalated $Ru(phen)_2dppz^{2+}$, with lifetimes of ~120 ns (85%) and ~700 ns (15%) in CT DNA (*84*). This long-lived excited state can be quenched by electron acceptors (*84*) and, though much less efficiently, by singlet oxygen (*16*). The oxidized form, $Ru(phen)_2dppz^{3+}$, has a reduction potential of 1.66 V (Table 1), allowing it to oxidize guanine (~1.3 V) (*8*).

$Ru(bpy)_3^{2+}$. $Ru(bpy)_3^{2+}$ has an MLCT excited state with a lifetime of ~600 ns (*74*) but is incapable of intercalating into the DNA base stack. With photophysical and redox properties similar to those of $Ru(phen)_2dppz^{2+}$, this molecule should reveal whether intercalative binding is necessary for cross-linking via the flash−quench reaction. It has a Ru^{3+}/Ru^{2+} potential of 1.56 V (Table 1), somewhat lower than

Table 2: Emission Quenching

photosensitizer	quencher	protein	% quenched	
acridine orange	$Co(NH_3)_5Cl^{2+}$	histone	55	*a*
ethidium	$Co(NH_3)_5Cl^{2+}$	histone	23	*a*
$Ru(bpy)_3^{2+}$	$Co(NH_3)_5Cl^{2+}$	histone	11	*a*
$Ru(phen)_2dppz^{2+}$	$Co(NH_3)_5Cl^{2+}$	histone	14	*a*
$Ru(phen)_2dppz^{2+}$	$Co(NH_3)_5Cl^{2+}$	cyt *c*	13	*a*
$Ru(phen)_2dppz^{2+}$	$Co(NH_3)_5Cl^{2+}$	BSA	19	*a*
ethidium	$Co(NH_3)_5Cl^{2+}$	histone	37	*b*
ethidium	$Ru(NH_3)_6^{3+}$	histone	77	*b*
ethidium	methyl viologen	histone	23	*b*

[a] Conditions: 10 μM photosensitizer, 100 μM quencher, 1 mM nuc CT DNA, and 250 μg/mL protein in a buffer of 10 mM NaP_i and 20 mM NaCl, pH 7. [b] Conditions: 25 μM ethidium and 250 μM quencher; other conditions same as in footnote *a*.

for $Ru(phen)_2dppz^{2+}$, and is a known sensitizer of singlet oxygen (*85*, *86*).

Acridine Orange. Acridine orange is an organic intercalator commonly used as a nucleic acid stain (*87*). Cyclic voltammetry of AO^{1+} gives an estimate of +1.47 V for the oxidation potential (Table 1), indicating that the 1-electron-oxidized form should be capable of oxidizing guanine. AO^{1+} emits a strong green fluorescence when intercalated into DNA and a strong red fluorescence when intercalated into RNA. The singlet excited state of the dye also undergoes a significant amount of intersystem crossing to the triplet state, making it an effective $^1O_2$ sensitizer (*88*−*90*).

Ethidium. Ethidium, an organic intercalator and common nucleic acid stain (*87*), has been shown to effect oxidation of guanine after oxidative quenching of the excited state (*39*). Here, the upper limit for the reduction potential of the ethidium dication was estimated to be +1.43 V (Table 1). Excited ethidium emits primarily out of the singlet state, with a fluorescence lifetime of ∼20 ns for the intercalated dye (*91*). The fluorescence is strongly quenched by water and other proton-accepting solvents (*92*, *93*).

The dependence of DNA−protein cross-linking yield on the identity of photosensitizer was determined by comparing $Ru(phen)_2dppz^{2+}$, $Ru(bpy)_3^{2+}$, acridine orange, and ethidium in flash−quench experiments. Figure 6 depicts the DNA−protein cross-linking trend for these photosensitizers in experiments utilizing CT DNA, histone 3-S, and $Co(NH_3)_5Cl^{2+}$. Samples were irradiated for times ranging from 0 to 6 min. For $Ru(phen)_2dppz^{2+}$, ∼25% of the DNA cross-links to protein within 15 s and ∼75% within 4 min of irradiation time. Samples containing $Ru(bpy)_3^{2+}$ exhibited slightly smaller amounts of cross-linking, with ∼20% at 15 s and ∼70% at 6 min. Utilizing acridine orange resulted in smaller yields of cross-link: ∼15% at 15 s and slightly more than 55% at 6 min. With ethidium as the intercalator, significantly less cross-linking was observed, with negligibly small amounts of cross-linking at 15 s and only ∼15% at 6 min of irradiation time. Therefore, the amount of cross-linking with respect to photosensitizer decreases in the order $Ru(phen)_2dppz^{2+}$ > $Ru(bpy)_3^{2+}$ > AO^{1+} > Et^{1+}. Control samples lacking photosensitizer show no evidence of cross-linking at either irradiation wavelength. Moreover, control samples lacking quencher do not exhibit significant cross-linking, with the exception of the acridine orange control, which shows up to 15% cross-linking at longer irradiation times. This latter result is consistent with the presence of competing reactions involving singlet oxygen.

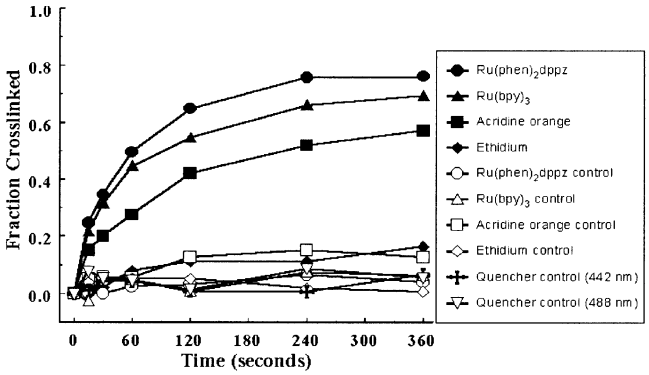


FIGURE 6: Dependence of DNA−protein cross-linking on the identity of the photosensitizer in the presence of $Co(NH_3)_5Cl^{2+}$ as quencher. Shown are data for samples containing $Ru(phen)_2dppz^{2+}$ (circles), $Ru(bpy)_3^{2+}$ (triangles), acridine orange (squares), and ethidium (diamonds), as well as respective control samples (open symbols) lacking quencher. Also shown are data for control samples for $Co(NH_3)_5Cl^{2+}$ in the absence of photosensitizer, irradiated at either 442 nm (crosses) or 488 nm (inverted triangles). Conditions: 10 μM photosensitizer, 100 μM $Co(NH_3)_5Cl^{2+}$, 1 mM nuc CT DNA, and 250 μg/mL histone in a pH 7 buffer of 10 mM NaP_i and 20 mM NaCl. Irradiations with a HgXe lamp (output ∼1 mW) were carried out at 488 nm for the acridine orange and ethidium samples and at 442 nm for the $Ru(phen)_2dppz^{2+}$ and $Ru(bpy)_3^{2+}$ samples. Control samples lacking intercalator were irradiated at each respective wavelength. The amount of DNA−protein cross-linking was determined by the chloroform extraction assay.

Given that the excited state lifetimes of the organic and inorganic photosensitizers are very different, it seemed logical to presume that differences in the quenching efficiencies might account for the observed cross-linking yields. Thus, the amount of steady-state emission quenching by $Co(NH_3)_5Cl^{2+}$ was measured for the four photosensitizers (Table 2). Surprisingly, the organic intercalators, despite their shorter excited state lifetimes, were quenched more efficiently than their inorganic counterparts.

Since the quenching results do not explain the cross-linking trend, we sought to determine whether differences in the next step, formation of guanine radical by oxidized photosensitizer, were responsible. Electrochemical measurements do indicate that $Ru(phen)_2dppz^{3+}$ is the strongest oxidant, and the oxidation potentials of the photosensitizers decrease in the order $Ru(phen)_2dppz^{2+}$ > $Ru(bpy)_3^{2+}$ > AO^{1+} and Et^{1+}. It should be noted that while the 1-electron oxidation of guanine to the cation radical may be thermodynamically favorable for all of the oxidized photosensitizers, the formation of the neutral guanine radical via a proton-coupled electron transfer process is probably more favorable still, as found in the electrochemical oxidation of guanine by $Ru(bpy)_3^{3+}$ (*94*). While the potentials do predict that $Ru(phen)_2dppz^{3+}$ will be the most effective oxidant, they alone do not reveal whether guanine oxidation actually occurs less readily with the other photosensitizers, since other factors such as binding geometry or nature of the excited state could play a role also.

One of the advantages of the flash−quench technique is that it permits the observation of intermediates as well as the permanent products formed from them, which would allow us to discern among reaction pathways of the different photosensitizers. DNA−protein cross-linking via the flash−quench technique is thought to occur via formation of a guanine radical intermediate. In the case of $Ru(phen)_2dppz^{2+}$,

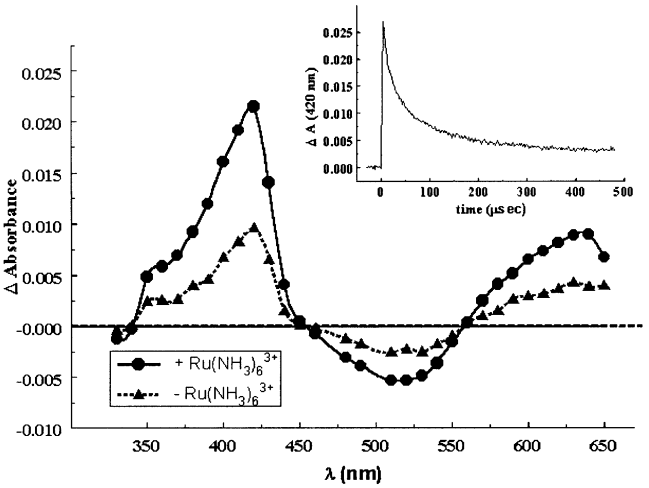


FIGURE 7: Spectra of long-lived transients formed upon photoexcitation of ethidium bound to poly(dG-dC). Shown are data for DNA-bound ethidium in the presence (circles, solid line) and absence (triangles, dashed line) of $Ru(NH_3)_6^{3+}$. Inset: signal at 420 nm for the sample containing $Ru(NH_3)_6^{3+}$. The individual signals at each wavelength were fit to a monoexponential decay function with a baseline offset to accommodate a very slow component: $\Delta A(t) = \Delta A(t{=}0) \exp(-kt) + \Delta A(\text{offset})$. For ethidium quenched by $Ru(NH_3)_6^{3+}$, the spectra of the fast and slow components were very similar, and the Δ absorbance values plotted here are for the dominant fast phase. For ethidium in the absence of $Ru(NH_3)_6^{3+}$, the spectrum shown is that of the slow component; the short-lived component in this case has a different spectrum (not shown) that resembles the ethidium triplet (see ref *95*). Conditions: 490 nm excitation (7 mJ/pulse), 30 μM ethidium, 3 mM nucleotides poly(dG-dC), and 0 or 600 μM $Ru(NH_3)_6Cl_3$ in an aerated buffer of 10 mM NaP_i and 20 mM NaCl (pH 7).

this intermediate is directly observable by transient absorption spectroscopy when the photosensitizer is quenched by $Ru(NH_3)_6^{3+}$ while bound to poly(dG-dC) (*37*). Moreover, the quenching process is rate-limiting for the formation of guanine radical in this GC DNA, since the rise of the guanine radical is concomitant with the decay of $*Ru(phen)_2dppz^{2+}$; no $Ru(phen)_2dppz^{3+}$ is detected (*37*).

Thus, transient absorption spectroscopy was used to determine if the other photosensitizers used here also generate an observable guanine radical. When ethidium is excited at 490 nm in the presence of poly(dG-dC) and $Ru(NH_3)_6^{3+}$, a long-lived transient[2] is formed (Figure 7, inset). The absorbance difference spectrum of this species has a maximum at 420 nm, a broad negative feature centered at about 500 nm, and positive features at wavelengths above 600 nm (Figure 7). This spectrum is similar to that of the ethidium dication formed by quenching of the ethidium triplet by oxygen (*95*, *96*). Figure 7 shows that the ethidium dication can be formed in the absence and presence of quencher, consistent with the findings of Atherton and Beaumont (*95*, *96*), but that the yield of 1-electron-oxidized ethidium is higher with the electron acceptor $Ru(NH_3)_6^{3+}$ present. Even though the ethidium dication is a fairly weak chromophore, no guanine radical signal, as characterized by broad maxima at 390 and 550 nm, was observed. This finding is in sharp contrast to the case of $Ru(phen)_2dppz^{3+}$, which gives rise to a guanine radical signal and is not even observable owing to its rapid reduction by guanine (*37*). Thus, since the ethidium dication is long-lived in poly(dG-dC), it appears that guanine is less efficiently oxidized by ethidium dication than by $Ru(phen)_2dppz^{3+}$.

Transient absorption spectroscopy was also used to detect transients formed with $Ru(bpy)_3^{2+}$ and acridine orange. Quenching of $*Ru(bpy)_3^{2+}$ by $Ru(NH_3)_6^{3+}$ in the presence of poly(dG-dC) gave rise to $Ru(bpy)_3^{3+}$, as suggested by long-lived negative signals around 440 nm, near the MLCT band of the complex (*74*). For acridine orange, the analogous flash−quench experiment gave rise to a long-lived transient, presumably from the triplet state since its presence did not require quencher; this transient was not the guanine radical because it did not exhibit a positive absorbance change in the 500−550 nm region. This latter result does not rule out formation of the guanine radical by acridine orange in the flash−quench reaction but suggests that any guanine radical that forms must decay more quickly than the acridine-based signal; the reduction of the guanine radical in poly(dG-dC) by the reduced quencher, $Ru(NH_3)_6^{2+}$, is mostly complete within 100 μs (*70*). While other signals interfered with the observation of the guanine radical with acridine orange and $Ru(bpy)_3^{2+}$ as photosensitizers, it should be pointed out that the guanine radical has been detectable with $Ru(phen)_2dppz^{2+}$ as photosensitizer even under conditions where Ru(III) (*42*) or another strong chromophore (e.g., Fe^{2+} cyt *c*) (*41*) was present. Thus, it is reasonable to state that the yield of guanine radical is indeed higher for $Ru(phen)_2dppz^{2+}$ than for the other photosensitizers. It is also reasonable to question whether these other photosensitizers do indeed oxidize guanine by flash−quench, and so we sought to demonstrate this experimentally.

Evidence for Selective Oxidation of Guanine by the Photosensitizers. It is well-known that the flanking sequence strongly influences the oxidation potential of guanine bases in DNA (*9*, *10*) and that the 5′ G of GG doublets is particularly susceptible to damage (*11*−*15*). To ascertain whether all of the photosensitizers employed here are capable of guanine oxidation, the sites of DNA damage in a mixed sequence oligonucleotide were examined by polyacrylamide gel electrophoresis. A 40-mer DNA strand containing three GG sites was chosen for study. The 40-mer was radioactively labeled with ^{32}P at the 5′ end, annealed to its complement, and then used in flash−quench experiments. Samples were then treated with hot piperidine, which results in strand breaks at the sites of modified bases (*4*). Figure 8 shows the gel electrophoretic analysis for the radiolabeled 40-mer run on a 15% polyacrylamide sequencing gel. Samples containing photosensitizer and quencher showed guanine-specific cleavage, whereas samples lacking light or photosensitizer or quencher gave minimal amounts of cleavage, indicating that the flash−quench pathway is operating to effect the damage. All of the photosensitizers showed a preference for cleavage at the 5′ G of GG sites, the signature for 1-electron oxidation (*11*−*15*); however, this preference was more pronounced for the metal complexes.

[2] The decay of the transient has two components. For ethidium quenched by $Ru(NH_3)_6^{3+}$, both components resemble the dication, with a maximum at 420 nm. However, for unquenched ethidium, the slow component resembles the dication and the faster component resembles the triplet state, with a maximum at 390 nm. This latter observation is consistent with the fact that the triplet is the precursor state to the dication when $Ru(NH_3)_6^{3+}$ is absent. That the fast component with $Ru(NH_3)_6^{3+}$ present has the spectrum of 1-electron-oxidized ethidium reflects the fact that, in this case, the oxidative quenching occurred on the much faster time scale of the fluorescence.

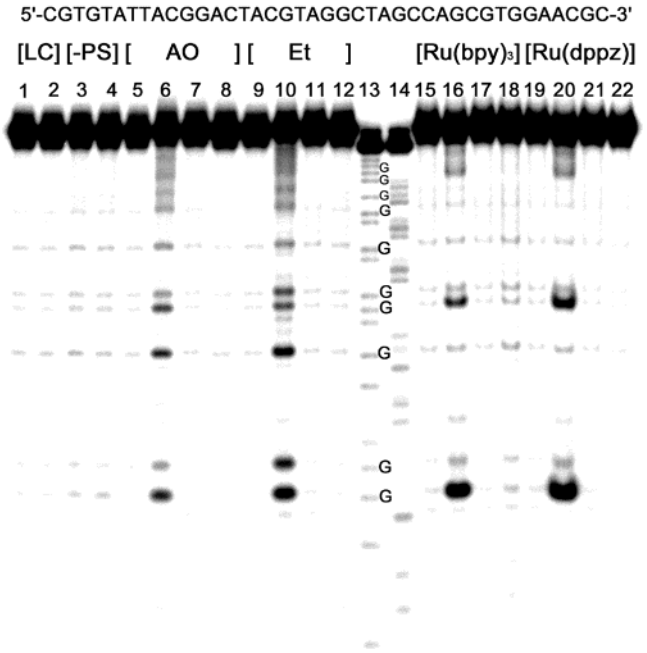


FIGURE 8: Oxidation and cleavage of a 5′-^{32}P-end-labeled 40 base pair oligonucleotide duplex via the flash−quench technique in the presence of different photosensitizers. Samples (30 μL) contained 20 μM photosensitizer, 0 or 200 μM cobalt quencher, and 25 μM oligonucleotide duplex in a buffer of 10 mM NaP_i and 20 mM NaCl (pH 7) and were irradiated with a HgXe lamp (output ∼1 mW). Samples containing acridine orange and ethidium were irradiated at 488 nm, whereas those with $[Ru(bpy)_3]^{2+}$ and $[Ru(phen)_2(dppz)]^{2+}$ were irradiated at 442 nm. All samples were treated with hot piperidine prior to analysis. Lanes 1 and 2: light controls (DNA only), irradiated at 442 nm (1 min) and 488 nm (10 min). Lanes 3 and 4: quencher controls, DNA + $[Co(NH_3)_5Cl]^{2+}$, irradiated at 442 nm (1 min) and 488 nm (10 min). Lanes 5 and 6: DNA + acridine orange + $[Co(NH_3)_5Cl]^{2+}$, irradiated for 0 and 10 min. Lanes 7 and 8: DNA + acridine orange, irradiated for 0 and 10 min. Lanes 9 and 10: DNA + ethidium + $[Co(NH_3)_5Cl]^{2+}$, irradiated for 0 and 10 min. Lanes 11 and 12: DNA + ethidium, irradiated for 0 and 10 min. Lanes 13 and 14: Maxam−Gilbert A+G and C+T sequencing reactions, respectively. Lanes 15 and 16: DNA + $[Ru(bpy)_3]^{2+}$ + $[Co(NH_3)_5Cl]^{2+}$, irradiated for 0 and 1 min. Lanes 17 and 18: DNA + $[Ru(bpy)_3]^{2+}$, irradiated for 0 and 1 min. Lanes 19 and 20: DNA + $[Ru(phen)_2(dppz)]^{2+}$ + $[Co(NH_3)_5Cl]^{2+}$, irradiated for 0 and 1 min. Lanes 21 and 22: DNA + $[Ru(phen)_2(dppz)]^{2+}$, irradiated for 0 and 1 min.

Detection of Ethidium-Induced Cross-Linking by Gel Shift Assay. Because ethidium is the most ubiquitous nucleic acid stain, its cross-linking reactions were characterized further. To detect the DNA−protein adducts, the gel shift assay (*79*) was employed. In this experiment, binding of cationic protein to DNA generates a complex that is larger and possesses a lesser negative charge than free DNA, resulting in a decrease in electrophoretic mobility for the DNA−protein complex. Figure 9 shows this gel shift assay applied to detect cross-linking between DNA and histone following quenching of $*Et^{1+}$ by $Co(NH_3)_5Cl^{2+}$. Lane 1, which contains only pUC19 DNA, shows two bands, the lower thicker band being supercoiled DNA and the higher thinner band being nicked DNA. Lanes 2 and 3 contain control samples in which photosensitizer and quencher were omitted from the flash−quench samples and show the same bands as lane 1. The samples in lanes 4−7, which contain Et^{1+}, $Co(NH_3)_5Cl^{2+}$, histone, and DNA, show a progressive change in the bands. As the irradiation time increases from 0 to 40 min, the intensity and mobility of the main band of supercoiled DNA decrease. Concomitantly, smearing between the nicked and supercoiled bands, and between these bands and the well, begins to appear due to mobility retardation caused by the protein, and a band begins to show up in the well. The sample in lane 8 is identical to that in lane 7 but has been digested with proteinase K. Lane 8 exhibits two bands near the bottom of the gel, with no smearing and no bands in the well. Since all of the cross-linked material disappeared upon treatment with proteinase K, the higher molecular weight species can be assigned to DNA−protein cross-links rather than DNA−DNA or DNA−ethidium cross-links.

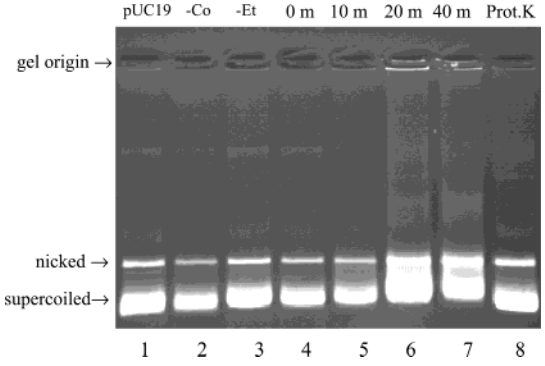


FIGURE 9: Cross-linking between pUC19 DNA and histone analyzed by agarose gel electrophoresis. Lane 1: pUC19 DNA only, irradiated for 40 min. Lane 2: pUC19 DNA, ethidium, and histone, irradiated for 40 min. Lane 3: pUC19 DNA, $Co(NH_3)_5Cl^{2+}$, and histone, irradiated for 40 min. Lanes 4−7: pUC19 DNA, ethidium, $Co(NH_3)_5Cl^{2+}$, and histone, irradiated for 0, 10, 20, and 40 min, respectively. Lane 8: pUC19 DNA, ethidium, $Co(NH_3)_5Cl^{2+}$, and histone, irradiated for 40 min and then digested with proteinase K (100 μg/mL) at 37 °C for 1 h. Samples were diluted with 10% SDS and loading buffer and run on a 0.8% agarose gel at ∼80 V for 2.5 h, with ∼1.5 μg of pUC19 DNA per well. Irradiated samples contained 10 μM ethidium, 100 μM $Co(NH_3)_5Cl^{2+}$, 1 mM nuc pUC19 DNA, and 250 μg/mL histone in a 10 mM NaP_i and 20 mM NaCl buffer at pH 7. Irradiation was carried out with 488 nm light (0.4 mW) from a 1000 W HgXe lamp.

Dependence of Ethidium-Induced Cross-Linking upon Quencher. For $Ru(phen)_2dppz^{2+}$ as the intercalating photosensitizer, it has been shown that the choice of quencher strongly affects the yields of both guanine damage (*37*) and DNA−protein cross-links (*70*). Thus, the dependence of cross-linking yield upon quencher, with ethidium as intercalator, was examined. Samples containing sonicated CT DNA and histone were irradiated at times ranging from 0 to 30 min in the presence of $Co(NH_3)_5Cl^{2+}$, MV^{2+}, and $Ru(NH_3)_6^{3+}$. Figure 10 shows the results of DNA−protein cross-linking detected by the chloroform extraction assay. Here, a stronger light source and higher concentrations of photosensitizer (25 μM) and quencher (250 μM) were employed to increase the efficiency of cross-linking and to make differences among the three quenchers more easily discernible. The sample containing ethidium and $Co(NH_3)_5Cl^{2+}$ showed the greatest fraction of cross-linking, reaching a value of ∼0.75 at 30 min of irradiation time; a control sample containing the cobalt complex but lacking ethidium exhibited no cross-linking over this time range. Samples containing ethidium and MV^{2+}, ethidium and $Ru(NH_3)_6^{3+}$, or ethidium without quencher (vide infra) showed significantly less cross-linking. It is worth noting that while ethidium alone produces a yield of cross-links comparable to that observed with $Ru(NH_3)_6^{3+}$ or MV^{2+} as quencher, the flash−quench pathway

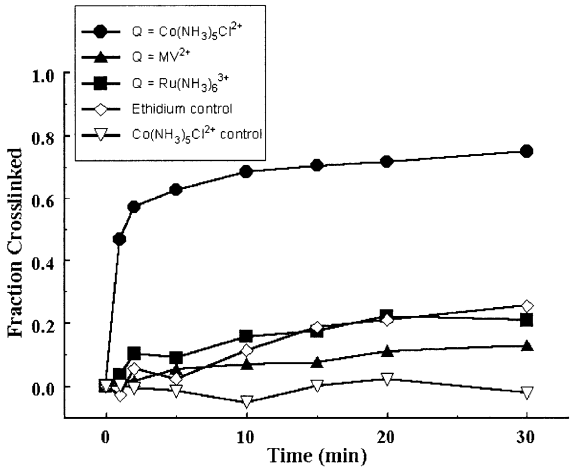


FIGURE 10: Dependence of DNA−protein cross-linking on the identity of quencher with ethidium as photosensitizer. Shown are data for samples containing $Co(NH_3)_5Cl^{2+}$ (circles), $Ru(NH_3)_6^{3+}$ (squares), methyl viologen (triangles), and control samples lacking quencher (diamonds) or photosensitizer (inverted triangles). Conditions: 25 μM ethidium, 250 μM quencher, and irradiation at 488 nm from a 1000 W Xe lamp (~3 mW); other conditions as in Figure 1. Note that the excitation output and the concentrations of photosensitizer and quencher are higher than in Figure 6.

is still a likely route to cross-linking with these quenchers.[3] Thus, with ethidium as photosensitizer, the cross-linking decreases in the order $Co(NH_3)_5Cl^{2+} \gg Ru(NH_3)_6^{3+} > MV^{2+}$. To determine whether the dependence of cross-linking yield upon quencher can be attributed to differences in quenching efficiency, fluorescence quenching measurements were carried out. For DNA-bound ethidium in the presence of histone, the emission was quenched 77% by $Ru(NH_3)_6^{3+}$, 37% by $Co(NH_3)_5Cl^{2+}$, and 23% by MV^{2+} (Table 2). Thus, the quenching efficiencies decrease in the order $Ru(NH_3)_6^{3+} \gg Co(NH_3)_5Cl^{2+} > MV^{2+}$, roughly the opposite of the trend in cross-linking.

DISCUSSION

Comparison of Photosensitizers. The data herein allow the evaluation of the different photosensitizers used in this study: $Ru(phen)_2dppz^{2+}$, $Ru(bpy)_3^{2+}$, acridine orange, and ethidium. All of these compounds can induce DNA−protein cross-linking via the flash−quench method, with the efficiency decreasing in the order $Ru(phen)_2dppz^{2+} > Ru(bpy)_3^{2+} > AO^{1+} > Et^{1+}$. Intercalation by the photosensitizer is not necessary, since the electrostatically bound $Ru(bpy)_3^{2+}$ can serve to induce cross-linking. Moreover, they all appear to do so via the specific oxidation of guanine (Figure 8), consistent with the fact that each of the oxidized photosensitizers is sufficiently high in potential to oxidize guanine (Table 1).

Interestingly, while all of the photosensitizers produced guanine-specific damage that was most pronounced at the 5′ G of GG doublets, there was clearly less 5′ G specificity for the organic photosensitizers. For the flash−quench samples of both acridine orange and ethidium, damage is evident at all guanines on the labeled strand, whereas damage from the ruthenium complexes is relatively restricted to the 5′ G of GG doublets. For the dppz complex of ruthenium, it has been observed that the guanine damage effected by flash−quench is almost completely localized at the 5′ G of the GG doublet (*16*). However, in studies of photoinduced guanine damage by ethidium using UV light, the piperidine-sensitive damage exhibited a weaker selectivity for the 5′ G of GG doublets than did a photooxidizing Rh(III) complex under similar conditions (*97*); acridine orange showed even less 5′ G specificity (*98*). Why the metal complexes should display a greater preference for oxidation of the 5′ G of GG doublets is not clear. The charge on the end of a DNA strand (*99*) and the presence of counterions (*100*) are thought to be important factors in DNA charge transport chemistry, so perhaps the higher charge on the metal complexes relative to the organic intercalators influences the trapping reaction of the guanine radical.

Our goal is to understand DNA−protein cross-links that occur from the reaction of proteins with guanine radicals. Despite the fact that all of these photosensitizers can function as cross-linking agents via the flash−quench technique, $Ru(phen)_2dppz^{2+}$ is clearly the best candidate for future studies for a number of reasons. First, $Ru(phen)_2dppz^{2+}$ is the most efficient cross-linking agent. Second, it is the only photosensitizer that generates an easily detectable guanine radical, allowing the intermediates of the reaction to be studied. Third, on the time scale of the experiments, $Ru(phen)_2dppz^{2+}$ does not react significantly with oxygen and only produces cross-links when the quencher is present. In contrast, acridine orange gives significant amounts of cross-linking in the absence of quencher (Figure 6), cross-linking that is eliminated upon the careful exclusion of oxygen (data not shown), and even ethidium gives some cross-linking at long irradiation times and higher concentrations (Figure 10). Both acridine orange and $Ru(bpy)_3^{2+}$, known to function as singlet oxygen sensitizers, generate more DNA damage via the flash−quench technique than in the absence of quencher, however. This finding is consistent with a study comparing the oxidation pathways of tryptophan, where it was found that the 1-electron oxidation of tryptophan by $Ru(bpy)_3^{3+}$ was more facile than oxidation by singlet oxygen generated by $*Ru(bpy)_3^{2+}$ (*101*). The authors concluded that type I (direct photooxidation) processes, of which flash−quench may be considered a subset, are likely to dominate over type II processes (singlet oxygen sensitization) when both pathways are competing. Given that our goal is to study cross-linking reactions that result from the formation of guanine radicals, $Ru(bpy)_3^{3+}$ is a less desirable oxidant than $Ru(phen)_2dppz^{3+}$ because, lacking intercalative binding, it is not as tightly bound to the DNA and could directly oxidize the protein instead. In summary, $Ru(phen)_2dppz^{2+}$ emerges as the best photosensitizer for making cross-links via guanine oxidation, owing to its efficiency of reaction, formation of detectable intermediates, and scarcity of competing reactions.

Dependence of Ethidium-Induced Cross-Linking upon Quencher. The trends in cross-linking and quenching efficiencies with quencher when ethidium is the photosensitizer are similar to those obtained previously for $Ru(phen)_2dppz^{2+}$

[3] At the higher concentrations used in Figure 10, ethidium induces cross-linking even in the absence of quencher. However, this cannot account for all of the cross-linking observed in the presence of $Ru(NH_3)_6^{3+}$ or MV^{2+} because the quenching process decreases the amount of $*Et^1$ present. For example, $*Et^{1+}$ is 77% quenched by $Ru(NH_3)_6^{3+}$, so if the cross-linking were occurring solely by an ethidium-only pathway, then the amount of cross-linking with $Ru(NH_3)_6^{3+}$ should be only 23% of that observed with ethidium alone. Instead, the amounts are similar, indicating that the flash−quench path must account for a substantial amount of the cross-linking when $Ru(NH_3)_6^{3+}$ is present.

(*70*). Flash−quench experiments with $Ru(phen)_2dppz^{2+}$ show that the amounts of guanine damage (*37*) and DNA protein cross-linking (*70*) decrease in the order $Co(NH_3)_5Cl^{2+}$ > MV^{2+} > $Ru(NH_3)_6^{3+}$, whereas the quenching efficiencies decrease in opposite fashion: $Ru(NH_3)_6^{3+}$ > MV^{2+} > $Co(NH_3)_5Cl^{2+}$. However, transient absorption measurements show that the guanine radical persists for >500 μs when $Co(NH_3)_5Cl^{2+}$ is the quencher and for only ~100 μs when $Ru(NH_3)_6^{3+}$ is the quencher, suggesting that the lifetime of the guanine radical is determining the yield of guanine damage (*70*). Although the guanine radical was not detected with ethidium, the cross-linking yields and quenching by $Co(NH_3)_5Cl^{2+}$ and $Ru(NH_3)_6^{3+}$ support this assertion: despite the fact that ethidium quenching by $Ru(NH_3)_6^{3+}$ is far more efficient, quenching by $Co(NH_3)_5Cl^{2+}$ leads to more cross-linking. In addition, quenching of ethidium by MV^{2+} gives rise to a similar amount of cross-linking as does quenching by $Ru(NH_3)_6^{3+}$, even though the metal complex is a much better quencher; this can be explained by the fact that quenching by MV^{2+} produces a longer lived guanine radical, owing to reduction of O_2 by MV^{1+} (*95*). Thus, for both $Ru(phen)_2dppz^{2+}$ and ethidium, the lifetime of the guanine radical appears to be the best predictor of the yield of DNA−protein cross-linking obtained with different quenchers.

Cross-Linking via the Flash−Quench Technique Does Not Involve Reactive Oxygen Species. The yield of cross-linking induced by quenching of $*Ru(phen)_2dppz^{2+}$ was shown to be unaffected by the absence of oxygen or by the presence of mannitol, a hydroxyl radical scavenger. Although we have not characterized products at this point, the insensitivity of cross-linking yield to oxygen rules out cross-linking via singlet oxygen or any other obligatory role for oxygen. That mannitol has no effect on cross-linking yield suggests that hydroxyl radicals are not important here either, although Eberhardt points out that it is not possible to definitively rule out site-specific hydroxyl radical formation in such experiments (*102*). However, we are confident that HO• is not involved, since our earlier studies showed that guanine bases are required for cross-linking via the flash−quench technique, while the highly reactive hydroxyl radical is not so discriminating and can attack all four of the DNA bases (*103*). Moreover, the sensitivity of the cross-linking to the piperidine treatment indicates formation of chemically modified bases, and the localization of the flash−quench-induced damage to GG sites also suggests that a 1-electron oxidation of guanine is occurring. Taken together, these results are consistent with cross-linking from a guanine radical intermediate formed by 1-electron oxidation rather than from reaction with reactive oxygen species. Nonetheless, it is important to note that since both the flash−quench technique and attack of HO• can result in formation of a neutral guanine radical (Scheme 1), oxidative stress in vivo could produce some of the same DNA−protein cross-links observed here.

Dependence of Cross-Linking upon Protein. Significant amounts of cross-linking were observed for histone and cytochrome *c*. That the trend in cross-linking (histone > cyt *c* > BSA) did not correlate with the quenching efficiencies indicates that the differences in cross-linking between the three proteins cannot be attributed to differences in quenching efficiencies. Instead, it is likely that the cross-linking trend observed simply reflects the fact that the cationic histone and cytochrome have a higher affinity for the DNA than does the anionic BSA. These results suggest that any number of basic proteins may be capable of reaction with guanine radicals. In vivo, however, where the ionic strength is higher than in our experiments, such cross-linking reactions are likely to be limited only to proteins that have a substantial affinity for DNA.

Cross-Linking vs Electron Transfer. Besides forming DNA−protein cross-links and other permanent lesions, the guanine radical is a strong oxidant that can also participate in electron transfer reactions. The peptides Lys-Trp-Lys and Lys-Tyr-Lys are known to reduce the guanine radical, and the radicals of the DNA-bound aromatic amino acids have been observed by transient absorption spectroscopy (*42*, *43*). Lys-Trp-Lys exhibited no cross-linking under these conditions (*42*). However, Lys-Tyr-Lys was found to cross-link with thymine residues of the DNA (*43*), presumably via the phenoxy radical, consistent with the findings of Dizdaroglu and others (*104*−*108*), who have characterized tyrosine−thymine cross-links. Here, we have also confirmed these two modes of reactivity for Fe^{2+} cytochrome *c*, which can both reduce the guanine radical (*41*) and cross-link with the DNA. Interestingly, Fe^{2+} and Fe^{3+} cytochrome *c* cross-link to the same extent with DNA, suggesting that Fe^{2+} cytochrome *c* prefers to react via cross-linking rather than electron transfer to the guanine radical. It was surprising to us that cross-linking competes so effectively with reduction of the guanine radical for Fe^{2+} cytochrome *c*. Perhaps adduct formation is facile because there is such a large number of potential reactive sites (e.g., lysines) on the protein for cross-linking, whereas electron transfer from Fe^{2+} cytochrome *c* to the guanine radical may require more specific binding orientations in which the heme−guanine distance is minimized. Since the rate for reduction of the guanine radical by Fe^{2+} cyt *c* is found to be $\sim 10^{10}$ M^{-1} s^{-1} (*41*), the *overall* rate constant for decay of the guanine radical to permanent damage products, such as cross-links or other lesions, must be larger. However, the reduced cytochrome could still influence the outcome of the cross-linking reaction: perhaps the ferroheme could reduce the initial adducts, likely radical species, into more stable products; the presence of antioxidants such as thiols does reduce the amount of 8-oxo-G formed upon exposure of DNA to oxidizing conditions (*109*, *110*). We are presently studying the interactions of small peptides and oxidized DNA with this effect in mind.

CONCLUSION

Herein, we demonstrate the power of the flash−quench technique as a method for generating DNA−protein cross-links that may result from oxidative stress in the cell. The flash−quench technique effects cross-links by a specific route, the 1-electron oxidation of guanine bases. However, the scope of this chemistry is more general, since flash−quench generates the neutral guanine radical, which is also formed when guanine encounters other common oxidants, such as the hydroxyl radical. The fact that several photosensitizers, including the widely used nucleic acid stains ethidium and acridine orange, can produce DNA−protein cross-links further reveals the versatility and general applicability of the flash−quench technique as a route to adduct formation. Moreover, the observation of cross-linking with cytochrome *c*, a nonphysiological binding partner of DNA, suggests that the range of possible proteins involved in

oxidative cross-linking reactions in vivo must include not only histones but other proteins that may be only transiently associated with the DNA, such as transcription factors and polymerases. Finally, these results illustrate the promise of the flash−quench technique for elucidating, at the molecular level, the products of cross-linking reactions of guanine radicals in double-stranded DNA.

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