Long-Range and Short-Range Oxidative Damage to DNA: Photoinduced Damage to Guanines in Ethidium−DNA Assemblies†

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ABSTRACT: Short-range and long-range photoreactions between ethidium and DNA have been characterized. While no DNA reaction is observed upon excitation into the visible absorption band of ethidium, higher-energy irradiation (313−340 nm) leads both to direct strand cleavage at the 5′-G of 5′-GG-3′ doublets and to piperidine-sensitive lesions at guanine. This reactivity is not consistent with oxidation of guanine by either electron transfer or singlet oxygen as shown by comparison with reactions of a rhodium intercalator and methylene blue, respectively. By covalently tethering ethidium to one end of a DNA duplex, we demonstrate the presence of two distinct reactions, one short-range and the other long-range. The short-range reaction involves a covalent modification of guanine by ethidium, based upon HPLC analysis of the nucleoside products and studies with ethidium derivatives. The long-range reaction is entirely consistent with oxidation of guanine by DNA-mediated electron transfer. The yield of this electron-transfer reaction is not attenuated with distance; equal yields of guanine damage are observed at a proximal (17 Å Et−GG separation) and distal (44 Å Et−GG separation) site. These results are quite similar to those previously observed with a covalently tethered rhodium photooxidant and underscore the unique ability of the DNA base stack to facilitate long-range electron transfer so as to effect oxidative damage from a distance.

Elucidation of the chemical mechanisms leading to DNA damage is critical for understanding the molecular basis of cancer and aging and will contribute to the development of new therapeutic strategies (*1*−*5*). Since the photoexcitation of fluorescent organic dyes can lead to the modification of DNA via cross-linking, electron transfer, and the generation of intermediate reactive species, including singlet oxygen and other radicals, these molecules have been employed extensively as mechanistic probes of DNA damage (*6*−*9*). Moreover, since many of the harmful effects of UV light are thought to be mediated by analogous natural sensitizers in vivo, information relevant to biological systems can be obtained by studying the photochemistry of organic molecules bound to nucleic acids in solution.

Metallointercalators can also be used to probe damage-causing processes in DNA (*10*−*14*). Rhodium intercalators promote direct strand cleavage by photoreaction with the deoxyribose unit proximal to the intercalation site (*15*), and ruthenium complexes can damage guanines in reactions mediated by singlet oxygen (*12*, *16*, *17*). Recently, in well-defined assemblies covalently modified with either rhodium or ruthenium intercalators (*10*−*12*), piperidine-sensitive base lesions were generated at sites more than 35 Å from the intercalating oxidants. These lesions result from oxidative damage to guanine through long-range electron transfer initiated from the stacked intercalators. Hence, these results provided the first demonstration of oxidative damage to DNA from a distance. Analogous observations were recently made using tethered anthraquinones as photooxidants (*18*). Indeed, it appears that DNA damage not only may be a localized event but also may be afforded by facile charge migration through the DNA base pair stack.

Interestingly, with *Rh(III) and Ru(III) intercalators (*10*−*13*), and with a variety of other reactants noncovalently bound to DNA that promote oxidative base damage via electron transfer, including anthraquinones (*19*, *20*), riboflavin (*21*), and naphthalimides (*22*, *23*), it is 5′-GG (underlined base denotes site of highest reaction) guanine doublet sites that are preferentially damaged. Ab initio calculations predict that the ionization potentials of guanine doublets are lower than that of any other dinucleotide sequence, and indicate that the HOMO for the dinucleotide is predominantly localized on the 5′-G (*24*). Hence, holes created by these photooxidants may migrate through the DNA base stack to sites which are thermodynamically favored. As a consequence, 5′-G specificity in reaction is becoming considered a signature of DNA damage occurring via electron transfer. This reactivity is easily distinguished from the more random, but typically guanine-specific reactivity of singlet oxygen sensitizers (*16*, *17*, *25*, *26*). Experimentally, this oxidative reaction must also be distinguished from the molecular recognition of 5′-GG-3′ doublets with subsequent base-independent reactivity.

To examine more fully the scope of DNA-mediated charge-transfer chemistry by intercalating photooxidants, we have characterized the photoreactivity of ethidium, a classical DNA intercalator, with DNA, and have systematically compared ethidium-promoted damage with oxidative damage by $Rh(phi)_2dmb^{3+}$ (phi is 9,10-phenanthrenequinone diimine and dmb is 4,4′-dimethyl-2,2′-bipyridine) (*10*−*11*) and

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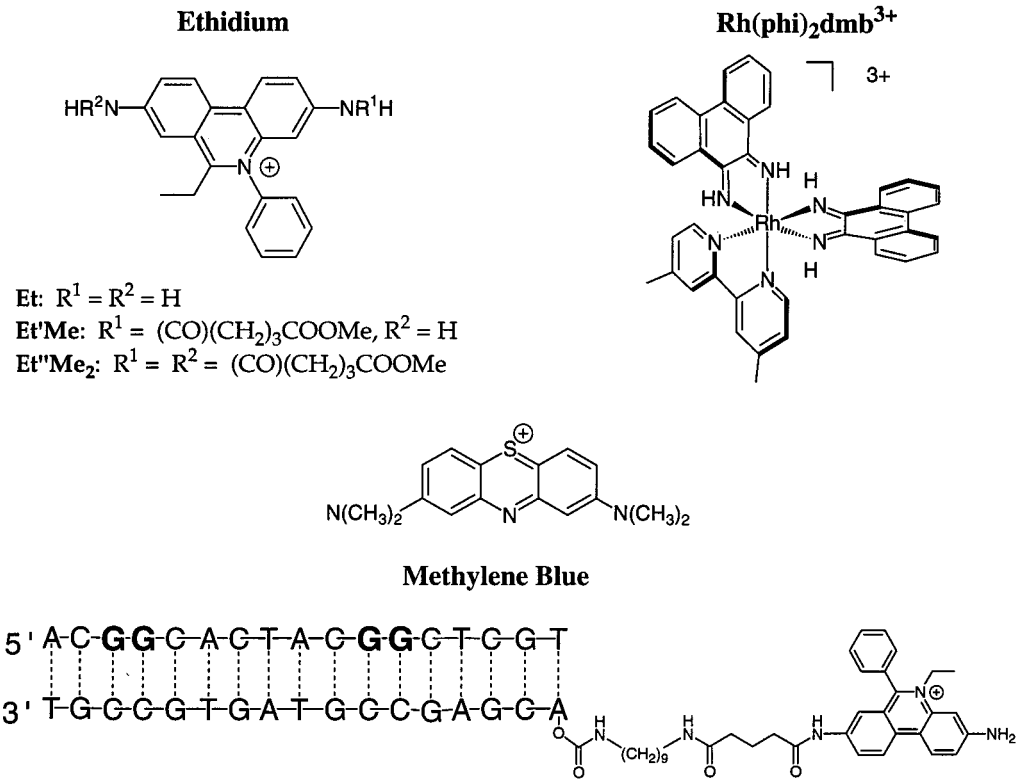

FIGURE 1: Intercalators (top) and Et′−DNA assembly (bottom) used for DNA photooxidation. The ethidium−DNA covalent assembly contains two potential 5′-GG-3′ sites of oxidation. If intercalation is assumed two base pairs from the end of the helix, the distance between the ethidium and the 5′-G of the proximal and distal 5′-GG-3′ site is 17 and 44 Å, respectively.

methylene blue, another intercalator which sensitizes $^1O_2$ (Figure 1). We find that ethidium, excited in the near-ultraviolet region, can promote both short-range DNA cross-linking and long-range oxidative base damage.

MATERIALS AND METHODS

Materials. Ethidium bromide and methylene blue were obtained commercially and used as received. [Rh(phi)$_2$dmb]-Cl_3 was synthesized according to published procedures (*27*). The synthesis of *N*-8-glycyl ethidium (Et′) has previously been reported (*28*). Bis-*N*,*N*-3,8-glycyl ethidium (Et″) is the major side product of this reaction. The methyl esters were obtained by refluxing the crude reaction mixture (0.05 g) in methanol (50 mL) in the presence of catalytic H_2SO_4 (20 μL). The products were purified by HPLC using a reversed phase C18 column (0 min, 100% NH_4OAc at pH 7; 50 min, 50% CH_3CN). The N-8-substituted methyl ester eluted first (46 min) followed by the N-3-substituted methyl ester (47 min) and the disubstituted methyl ester (50 min).

Oligonucleotides were assembled on an ABI synthesizer and purified by C18 reversed phase HPLC both before and after the removal of the trityl protecting group. Rhodium-modified (*10*) and ethidium-modified (*28*) DNA oligonucleotides were synthesized by published procedures. The following unmodified or modified (**X** = Rh′ or Et′) duplexes were constructed (* = ^{32}P): **X**- -ACGAGCCGTAGTGC-CGT-3′ and *5′-ACGGCACTACGGCTCGT-3′. Studies in which this rhodium-modified assembly was used have previously been reported (*11*). The identity of the single-stranded Et′−DNA conjugate was verified by ESI mass spectrometry: MW 5804.8 (found) and 5805.2 (calculated).

DNA Techniques and Photocleavage Experiments. The DNA strand containing the 5′-GG-3′ sites was 5′-^{32}P-end-labeled using polynucleotide kinase and purified on a 10% denaturing polyacrylamide gel. The labeled DNA was separated from the polyacrylamide by soaking the crushed gel pieces in 500 μL of TE buffer [10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8)] at 37 °C for 4 h followed by spin filtration. The labeled DNA was desalted on a Nensorb 20 DNA purification cartridge (DuPont) by washing the DNA with water (1 mL) before eluting with 1:1 water/acetonitrile (1 mL). Samples were annealed on a thermocycler by heating at 90 °C for 5 min and then cooling to 20 °C over the course of 70 min. The noncovalently bound intercalators are added after the annealing process is complete.

Irradiations were performed on 20 μL samples in 1.5 mL Eppendorf tubes using a 1000 W Hg/Xe lamp equipped with a monochromator. The concentrations and buffers used are indicated in the figure captions. After irradiation, samples or portions of samples that did not require piperidine treatment were dried under vacuum. Piperidine treatment consisted of adding a freshly prepared solution of water and piperidine such that the total volume was 100 μL and the final piperidine concentration was 10% (1 M). After they were heated for 30 min on a 90 °C heat block, the samples were immediately dried under vacuum and twice resuspended in water (20 μL) and redried to remove trace amounts of piperidine. The radioactivity was measured on a scintillation counter, and 80% formamide dye was added such that each sample had the same number of counts per microliter. The samples were analyzed by 20% denaturing polyacrylamide gel electrophoresis followed by phosphorimagery.

D_2O/H_2O Experiments. Two different deuterated buffers were utilized. The first was prepared by dissolving sufficient ammonium acetate in D_2O to yield a 1 M solution, resulting in a pH of 7.2 which was not adjusted. The second was prepared with the proper ratio of Na_2PO_4 and $NaHPO_4$ to yield a 50 mM solution with a pH of ~7. The pH was adjusted to 7.0 by adding small amounts of Na_2PO_4 or $NaHPO_4$. Analogous buffers were prepared with H_2O as the solvent. The two strands of DNA and the ^{32}P-labeled DNA were prepared in H_2O and dried under vacuum. D_2O was added, and the samples were dried again. Then, H_2O/D_2O and buffer (25 mM ammonium acetate or 15 mM sodium phosphate and 50 mM NaCl) were added and the samples annealed (90 °C for 5 min, cooled to 20 °C over the course of 70 min). Finally, a solution of the intercalator in either H_2O or D_2O was added.

$N_2/Air/O_2$ Experiments. Irradiation sample volumes were either 50 μL (covalently modified duplexes) or 100 μL (unmodified duplexes). Samples were sealed inside small glass vials equipped with septa and frozen in liquid nitrogen and the vials evacuated for 30 s followed by addition of the appropriate gas. This was repeated twice, and the samples were thawed. Then, the entire freeze−pump−thaw procedure was repeated, and irradiations were performed after the samples had warmed to room temperature.

Base Analysis. Irradiations were performed for 6 h on 300 μL samples in quartz cells with the contents being stirred constantly. The samples were then diluted 2-fold with water and sodium acetate (pH 5, final concentration of 20 mM) and digested with nuclease P_1 (20 μL, 0.6 unit/μL, stored in 10 mM NaOAc, pH 5) at 37 °C. After 2 h, 1.0 M Tris buffer (20 μL, pH 7.5) and alkaline phosphatase (3 μL) were added, and the mixture was incubated for an additional 2 h at 37 °C. These samples were injected directly onto the HPLC system. The HPLC conditions were taken from Ito et al. (*21*). A flow rate of 0.8 mL/min through a Microsorb 100 Å C18 reversed phase column was used on a Waters HPLC system driven by Millennium software.

RESULTS

Photoinduced Cleavage of DNA by Noncovalently Bound Intercalators. To distinguish between different modes of DNA damage, three intercalators (Figure 1) were irradiated bound to a ^{32}P-end-labeled 17 bp DNA oligonucleotide duplex (Figure 2) and examined by denaturing gel electrophoresis. With 313 nm as a starting point, wavelengths out into the visible region were chosen to match the absorption spectrum of each compound. The sites of direct DNA strand breakage versus sites of piperidine-labile damage are compared in panels A and B of Figure 2, respectively, for Rh(phi)$_2$dmb^{3+}, ethidium bromide, and methylene blue. Extensive, nondiscriminate DNA cleavage is observed without piperidine treatment across the oligomer upon irradiation of Rh(phi)$_2$dmb^{3+} at 313 nm in the presence of duplex DNA. These strand breaks have previously been shown to yield products consistent with abstraction of a 3′-hydrogen atom from neighboring sugar residues at sites of intercalation (*15*, *29*).

Ethidium also promotes direct strand cleavage but with a pronounced specificity for 5′-GG-3′ sites upon irradiation at 313 nm and to a lesser extent at 340 nm. It should be noted that the yield of direct cleavage at guanine with ethidium irradiation at 313 nm is sensitive to the buffer. As shown, in sodium cacodylate buffer, significant direct cleavage is obtained. The yield, however, is lower with an ammonium acetate buffer. The piperidine-sensitive lesions (vide infra) do not appear to be affected by the choice of buffer.

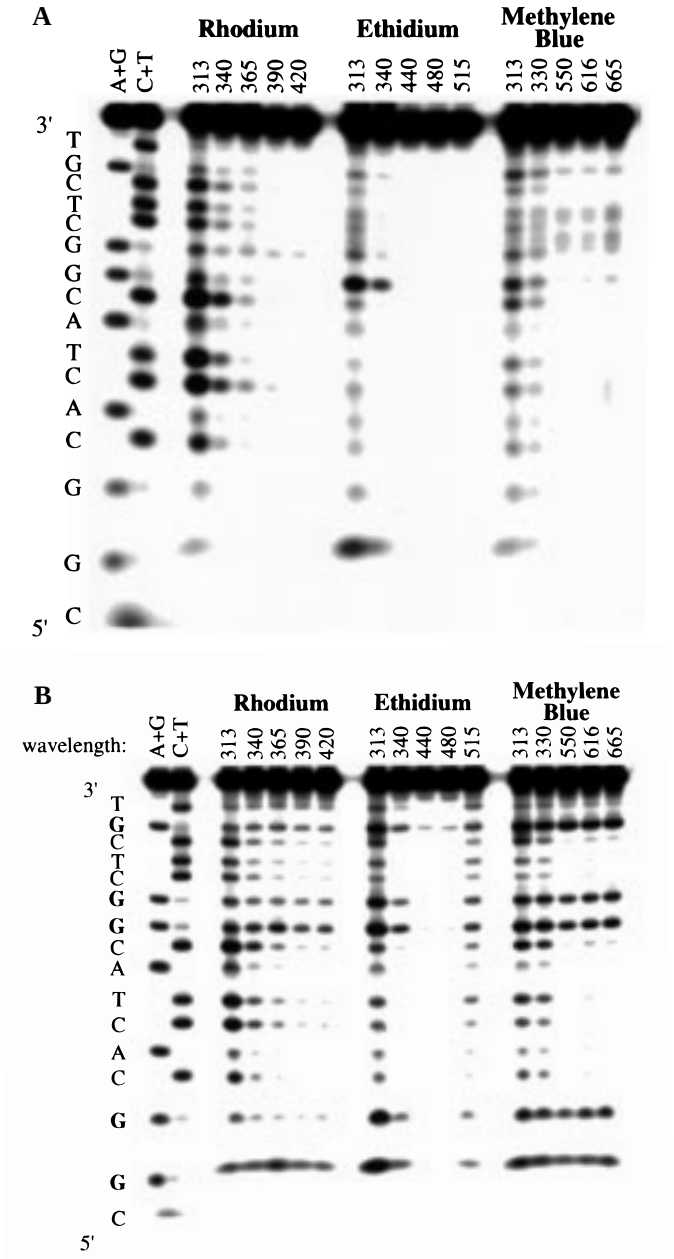

FIGURE 2: Direct photocleavage (A) and piperidine-induced cleavage (B) of DNA with Rh(phi)$_2$dmb^{3+}, ethidium, and methylene blue as a function of wavelength. The five wavelengths examined for each compound are listed at the top of each lane. Each sample was irradiated for 15 min except for Rh(phi)$_2$dmb^{3+} at 313 nm which was irradiated for 5 min. The Maxam−Gilbert A+G and C+T sequencing lanes are shown on the far left. The following concentrations were used: 10 μM duplex DNA, 25 μM intercalator, and 25 mM sodium cacodylate buffer (pH 8). The sequence of the 5′-^{32}P-labeled 17-mer oligonucleotide is shown on the left. Half of each irradiation sample was analyzed directly as shown in panel A, and half was treated with hot piperidine before gel electrophoresis as shown in panel B. No cleavage was detected upon irradiation of oligonucleotides in the absence of intercalators.

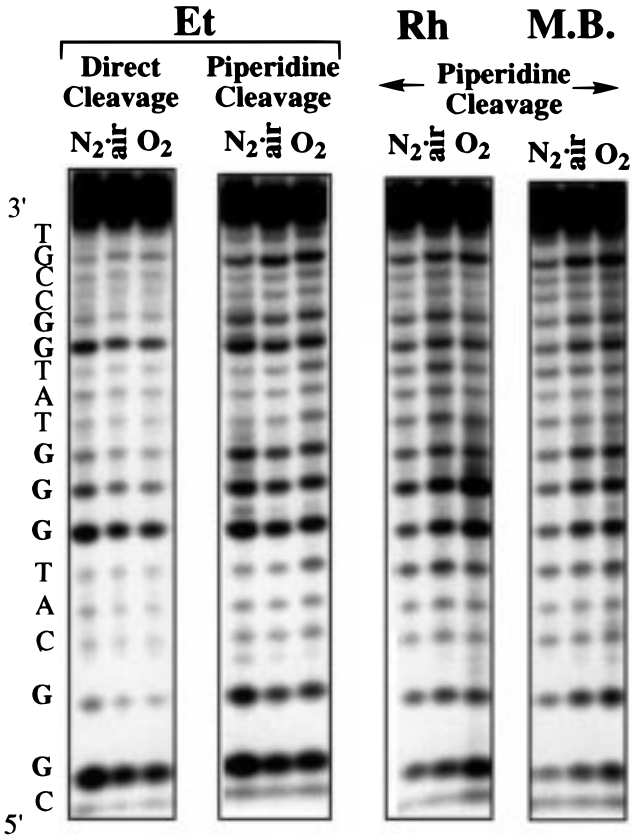

FIGURE 3: Photoinduced damage of guanine by ethidium, Rh(phi)$_2$dmb^{3+}, and methylene blue in N_2, air, or O_2. All samples were degassed thoroughly as described in Materials and Methods. Both the direct and piperidine-induced cleavage are shown for ethidium (Et); only the piperidine-treated samples are shown for rhodium (Rh) and methylene blue (M.B.). The samples were irradiated for 20 min at 313 (ethidium and methylene blue) or 365 nm [Rh(phi)$_2$dmb^{3+}]. Irradiation samples consisted of 10 μM duplex DNA, 25 mM sodium cacodylate buffer (pH 8), and 25 μM intercalator.

Excitation of methylene blue bound to DNA also causes direct strand cleavage but with little sequence specificity; a small preference for guanine is noteworthy. The products of these reactions all comigrate with the Maxam−Gilbert sequencing lanes, indicating the presence of 3′-phosphate termini.

Piperidine treatment reveals extensive damage to the guanine bases in each of these photoreactions (Figure 2B). For Rh(phi)$_2$dmb^{3+}, this damage occurs maximally with irradiation at 365 nm and shows a pronounced 5′-GG-3′ specificity. This guanine doublet-specific damage is superimposed over that observed without piperidine treatment, indicating the presence of two distinct reactions. The direct strand cleavage reaction with guanines by ethidium is, in contrast, apparently enhanced by piperidine treatment. It is noteworthy, however, that the piperidine-sensitive damage generated with 313 excitation of ethidium shows a smaller degree of specificity than that found with direct cleavage, whereas excitation with 340 nm reveals 5′-GG-3′ discrimination comparable to that found with Rh(phi)$_2$dmb^{3+}. Methylene blue irradiation promotes piperidine-labile lesions at every guanine.

Effect of Solvent and O_2 on Photocleavage Reactions. Figure 3 illustrates the effect of O_2 on these cleavage reactions. As previously reported (*10*), piperidine-sensitive guanine damage by Rh(phi)$_2$dmb^{3+} requires O_2. Direct strand cleavage is not O_2-dependent, however. A dependence of piperdine-sensitive reaction on O_2 is found also for methylene blue (Figure 3). Interestingly, the extent of direct cleavage by ethidium is substantially diminished in the presence of oxygen (4-fold). The piperidine-sensitive damage, once the direct reaction is accounted for, appears to be unaffected by O_2.

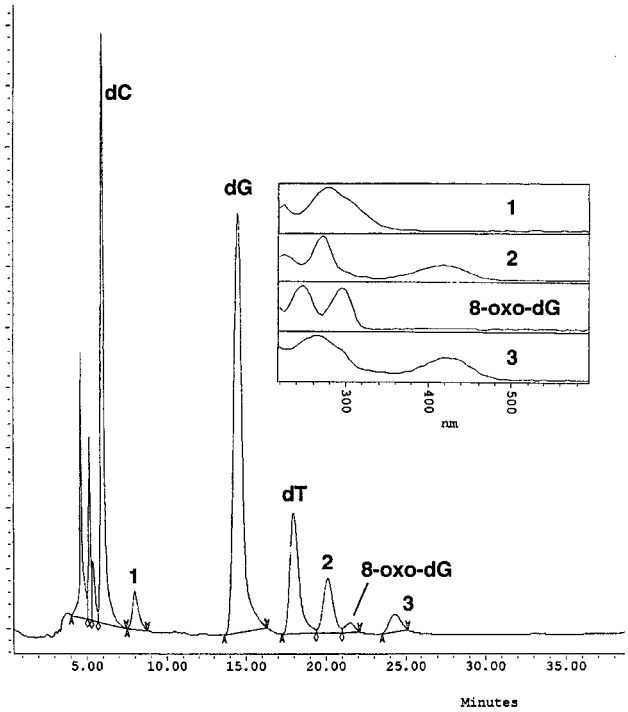

FIGURE 4: HPLC determination of DNA base damage induced by ethidium and 313 nm light. Three well-resolved peaks in traces monitored at 300 nm that did not correspond to injected nucleoside standards are numbered along with their corresponding absorption spectra (inset). A small amount of 8-oxo-dG is also detected. The sample consisted of 300 μL of 20 μM duplex DNA, 100 μM intercalator, and 25 mM sodium cacodylate buffer (pH 9).

The effect of D_2O on the yield of guanine damage was also examined. As expected for a singlet oxygen sensitizer (*30, 31*), the yield of damage increases approximately 2-fold for methylene blue (data not shown). In contrast, no D_2O effect was observed for rhodium or ethidium.

Product Analysis. Modified bases were detected by HPLC analysis of the nucleosides obtained by digestion with nuclease P_1 and alkaline phosphatase. The presence of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) is easily detected by its characteristic absorption at 300 nm. The formation of 8-oxo-dG by Rh(phi)$_2$dmb^{3+} has been shown to correlate with the number of piperidine-induced strand breaks (*30*). Figure 4 shows the HPLC analysis of DNA products after irradiation in the presence of ethidium at 313 nm and subsequent enzymatic digestion. Significantly less 8-oxo-dG is produced in the presence of ethidium compared to that with methylene blue and Rh(phi)$_2$dmb^{3+}. However, significant amounts of other unique products are detected for the ethidium reaction. These products show characteristic blue-shifted ethidium-like absorptions around 400 nm, but contain other spectral features in the ultraviolet region distinct from those of ethidium. It should be noted that these products are not observed when ethidium is irradiated in the absence of DNA.

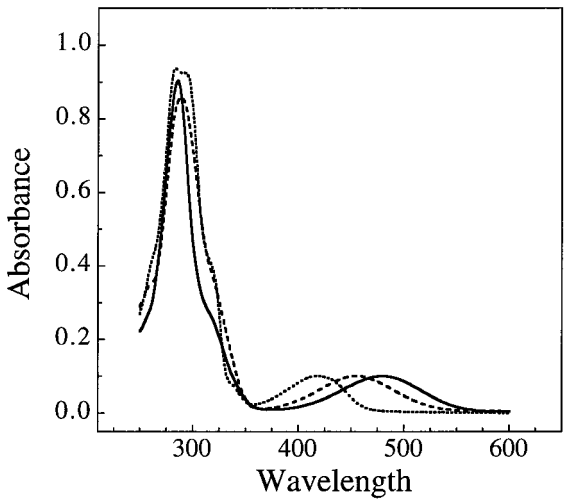

FIGURE 5: Absorption spectra for ethidium (solid line) and modified analogues Et′-Me (dashed line) and Et″-Me_2 (dotted line) obtained in H_2O.

Comparison of Reactivities for N-Alkylated Ethidium Derivatives and Ethidium-Modified Oligonucleotides. The relative cleavage efficiencies and specificity of guanine damage were also investigated for a family of N-alkylated ethidium derivatives and an ethidium-modified DNA duplex (Figure 1). For the modified ethidium analogues, the carboxylic acid of the N-alkyl chain was esterified to preserve the positive charge on each molecule. Although modification of the exocyclic amines dramatically affects the energy of the visible absorption band, it has a smaller effect on the shape of the spectrum in the UV region (Figure 5), and thus, irradiations were performed at identical wavelengths.

Distinct photoreactivities are evident with and without piperidine treatment (Figure 6). The singly modified ethidium derivative (Et′-Me) displays identical reactivity upon photolysis in the presence of DNA found with unsubstituted ethidium, although the overall efficiency is lower. For both, more extensive reaction is apparent with piperidine treatment. Strikingly, the disubstituted ethidium derivative (Et″-Me_2) yields no direct cleavage with irradiation. Piperidine treatment, however, yields distinctive lesions at 5′-GG-3′ sites after irradiation at 313 nm in the presence of Et″-Me.

Importantly, when ethidium is tethered to DNA, short-range and long-range photoreactions are separated. If direct strand cleavage is monitored, damage only at the single guanine near the tethered end of the duplex is observed; no direct cleavage at the more remote 5′-GG-3′ sites is observed. In contrast, upon piperidine treatment, cleavage is detected at the 5′-guanine of both 5′-GG-3′ doublets.

As established earlier for tethered rhodium species, the reaction occurs in an intraduplex fashion. Irradiation of the unlabeled Et′−DNA duplex in the presence of ^{32}P-end-labeled DNA lacking ethidium yields neither direct nor piperidine-sensitive damage. The intraduplex reaction shown in Figure 6 therefore corresponds to oxidative damage to the two 5′-GG-3′ doublets by photoexcited ethidium being promoted over a distance of 17 and 44 Å in these tethered assemblies.

Figure 7 illustrates several characteristics of this long-range electron-transfer reaction. Unlike the situation with noncovalently bound ethidium, a dependence of the piperidine-sensitive reaction on O_2 is evident. It should be noted that

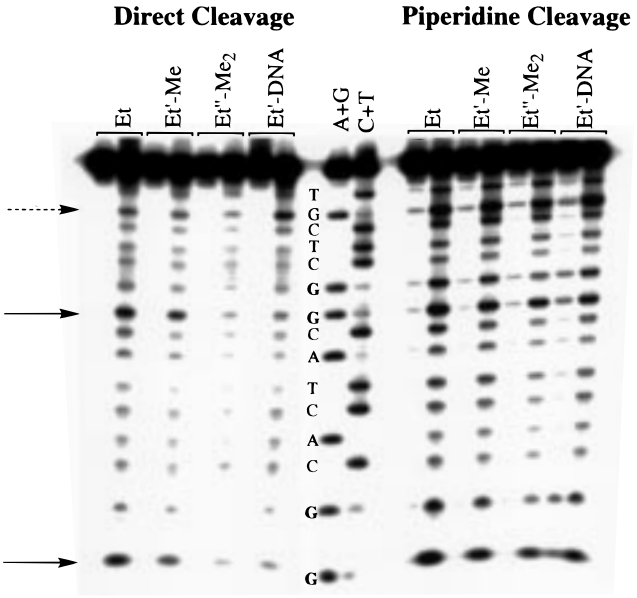

FIGURE 6: Comparison of 313 nm photocleavage with ethidium (Et), the singly substituted ethidium (Et′), the disubstituted ethidium (Et″), and the ethidium−DNA conjugate (Et′−DNA). The pattern of direct cleavage is shown on the left side and that of piperidine-induced cleavage on the right. Each set of two lanes contains the 0 and 30 min irradiations from left to right. Irradiation samples consisted of 20 μM duplex DNA, 20 μM ethidium, and 25 mM sodium cacodylate buffer (pH 8). The 5′-guanines of the two 5′-GG-3′ sites are indicated with solid arrows, and the single guanine near the end to which the ethidium is covalently tethered in the Et′−DNA conjugate is shown by a dashed arrow.

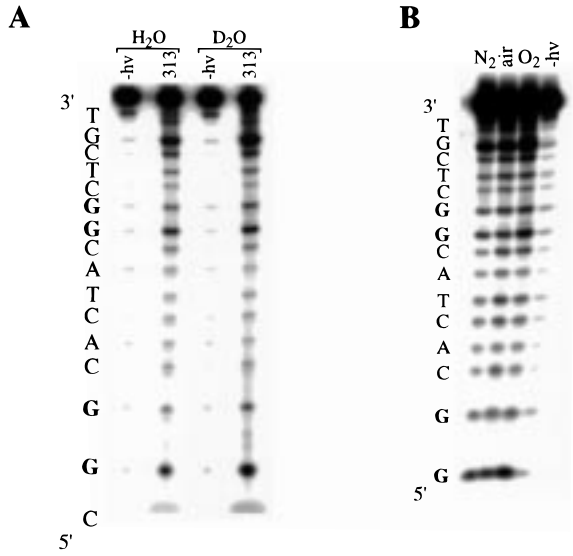

FIGURE 7: Effect of D_2O (A) and O_2 (B) on 313 nm photocleavage by Et′−DNA. (A) An unirradiated (−*hν*) and 313 nm irradiated sample are shown for both H_2O and D_2O. Each sample contains 15 μM Et′−DNA, 15 mM sodium phosphate buffer (pH 7), and 50 mM NaCl and was treated with hot piperidine after irradiation. (B) Samples were irradiated for 1 h in a nitrogen (N_2), air, or oxygen (O_2) atmosphere. Also shown is an unirradiated sample (−*hν*). Samples were subjected to hot piperidine prior to analysis by gel electrophoresis and contained 15 μM Et′−DNA and 25 mM sodium cacodylate buffer (pH 8).

small amounts of damage occur even in the N_2 atmosphere; a low level of O_2 contamination cannot be ruled out, however. This overall sensitivity in the long-range oxidative damage matches that previously seen with rhodium (*10*). Thus, it appears that while the direct cross-linking reaction

between ethidium and DNA is more efficient without oxygen, the long-range oxidative damage generated required oxygen, likely as a trapping agent. Furthermore, with tethered ethidium, HPLC analysis detects the presence of 8-oxo-dG after enzymatic digestion without piperidine, as identified by coelution and spectral comparison with a known standard (data not shown). The yields of piperidine-sensitive damage observed with covalently tethered ethidium and rhodium are found to be comparable. In fact, the overall quantum yield then is somewhat higher for ethidium, given its lower extinction coefficient and the lower photon flux at 313 versus 365 nm. In contrast to results with rhodium, with tethered ethidium, a small D_2O effect at each guanine is observed (Figure 7); at the proximal 5′-GG-3′ doublet, the enhancement is by a factor of 1.4, while at the distal site, the enhancement is 2-fold.

DISCUSSION

High-Energy Photoreactivities of Ethidium. Ethidium is typically utilized as a probe of nucleic acid structure and ubiquitous nucleic acid stain, since the fluorescence generated with visible excitation is strongly enhanced upon intercalation of this heterocycle into DNA (*32*). The increased quantum yield and lack of reactivity with natural nucleotides indicate (*33*) that the lowest singlet state [$E°(*Et/Et^-) = +1.1$ V vs NHE (*28*)] is not sufficiently energetic to undergo electron transfer with the DNA bases. However, upon irradiation with higher-energy light ($\lambda = 310-340$ nm) that corresponds to the shoulder of the main UV absorption band for ethidium, our results establish that a more reactive state is generated that appears to be capable of both oxidizing guanine and reacting directly with this base. The operative excited state may represent the second singlet transition for ethidium; it has been calculated that this state would be ~0.9 eV higher in energy [$E°(*Et/Et^-) > 2$ V] than the first singlet excited state (*34*). It is important to note that an electron-transfer reaction with this species must occur within the lifetime of this high-energy excited state which is likely to be very short. One other laboratory has previously studied DNA photocleavage of ethidium−DNA conjugates (*35*). In this case, a high-energy 337 nm laser was used to achieve a highly reactive two-photon absorption excited state which leads to direct cleavage and cross-linking only near the site of attachment. Other experiments with excitation of ethidium in the visible region required methyl viologen as an added quencher to generate guanine-specific cleavage (*36*).

Direct Strand Cleavage by Ethidium. Direct strand cleavage is observed upon irradiation of ethidium at 313 and 340 nm. The specificity for cleavage at 5′-guanines of 5′-GG-3′ sequences suggests the involvement of electron-transfer chemistry. However, unlike the damage caused by most other agents known to oxidize guanines, piperidine treatment is not required for the strand breaks. Although extensive direct photocleavage of DNA by ethidium upon 514 nm irradiation has been reported (*37*), we do not see evidence for this at 520 nm.

HPLC analysis of the nucleosides generated during the photoreaction of ethidium with DNA provides evidence for a covalent reaction between this intercalator and the DNA bases. Detection of products with visible absorptions similar to that of ethidium and ultraviolet absorptions resembling guanine and 8-oxoguanine suggests that a direct reaction between the fluorophore and an adjacent guanine may produce a covalent adduct. For this product, the shift of the visible absorption peak to a higher energy is consistent with modification of the exocyclic amine of ethidium (see Figure 5). The direct reaction of ethidium with guanine upon photoexcitation after an electron-transfer event would explain the 5′-G specificity of this reaction. Irreversible formation of a ruthenium−guanine adduct has been observed following photoinduced guanine oxidation (*38*, *39*), and the guanine radical has been shown to be susceptible to nucleophilic attack (*40*, *41*). The enhancement of DNA cleavage by ethidium in nitrogen may provide further evidence that the intercalator competes with oxygen in the trapping of the guanine radical.

Because these ethidium−DNA adducts were detected, it seemed logical that the operative mechanism required direct contact between the ethidium and the target guanine, and possibly involved addition of the exocyclic amine to the guanine (vide supra). To investigate this further, the photocleavage of DNA by ethidium derivatives which had been modified at one or both of the exocyclic amines was investigated. The singly modified ethidium derivative (Et′-Me), which is the chromophore analogous to that employed in Et-modified duplexes, displayed similar reactivity but reduced efficiency when compared to unmodified ethidium, initiating both direct strand breaks and piperdine-sensitive lesions. In contrast, the doubly modified Et″-Me_2 yielded no direct cleavage but instead induced piperidine-sensitive damage at 5′-GG-3′ sites only. Thus, while the short-range mechanism is inoperable, Et″-Me_2 is still able to induce oxidation in a manner indicative of electron transfer with guanine. This supports the notion that the exocyclic amines are critical for the short-range reaction which may lead to direct strand breaks.

Oxidation of Guanine by Ethidium. Although ethidium yields direct strand breaks with a high degree of 5′-guanine specificity upon irradiation at 313 nm, the additional cleavage caused by piperidine occurs at every guanine (Figure 2B). However, at 340 nm, greater 5′-G specificity is observed for the piperidine-sensitive cleavage. These differences in reactivity may reflect energetic differences in the states generated by these excitation wavelengths, with higher-energy irradiation potentially leading to reaction with sites with higher oxidation potentials. The guanine damage induced by noncovalently bound ethidium is not enhanced by D_2O. In addition, cleavage occurs preferentially in the absence of oxygen. As expected (*37*), these observations clearly rule out a reaction mediated by singlet oxygen. The substantial differences in pattern and characteristics of reactivity between ethidium, ethidium derivatives, and methylene blue, a well-known singlet oxygen sensitizer (*30*, *31*), underscore the idea that $^1O_2$ does not contribute to ethidium reactivity. Previous studies of singlet oxygen sensitization by this intercalator have also shown that production of this reactive species is simply not efficient for DNA-bound ethidium (*32*, *42*). Instead, the concentration of damage at signature 5′-GG-3′ sites with 340 nm excitation provides evidence that this intercalator can effect photoinduced damage of guanine via electron transfer.

Long-Range Oxidation of Guanine Doublets by Ethidium. If photolysis of ethidium can promote electron-transfer

chemistry with DNA bases, then the ability of ethidium to oxidize guanine doublets from a remote position along the DNA helix could also be tested in a DNA assembly containing this intercalator tethered and therefore spatially separated from a 5′-GG-3′ doublet. Strikingly, no direct strand breaks are formed at the 5′-GG-3′ sites, only piperidine-labile lesions (Figure 6). Importantly, the only site of direct cleavage observed for the Et−DNA conjugate is the single guanine near the tethered end of the duplex. This cleavage pattern is fully consistent with the expectation that tethered ethidium is confined to this end of the DNA helix. Indeed, the reaction serves to mark the site of association. However, piperdine-sensitive lesions are generated at positions 17 and 44 Å away from the tethered intercalator, indicating that oxidative damage generated by this species can migrate over extended distances. The long-range reactivity observed with ethidium is analogous to that obtained with tethered rhodium, where (i) a strong 5′-G specificity requiring piperidine treatment for strand scission is observed, (ii) O_2 is required, and (iii) 8-oxo-dG is a major product as revealed by HPLC (*30*).

The guanine oxidation reactions in covalently modified ethidium and rhodium duplexes do, however, differ with respect to solvent effects. For the ethidium-modified assembly, the yield of damage is modestly increased in D_2O. The increase at the distal site is equal to or slightly greater than that at the proximal site. If singlet oxygen were involved, the proximal site should be preferentially affected. Experiments conducted in our laboratory have shown that when a ruthenium complex is tethered to one end of a DNA helix, singlet oxygen damage is limited to less than five base pairs from the end (*12*). It is noteworthy that the excited state lifetime (for the first excited state) of ethidium, unlike that of rhodium, is increased 2-fold in D_2O (*32*); the effect of D_2O on the higher-energy excited state responsible for the chemistry here is not known.

Comparison of Singlet Oxygen Sensitization versus Electron-Transfer Chemistry. DNA oxidation via electron transfer and reactions with singlet oxygen both lead to the piperidine-sensitive lesions at guanine which are typically associated with the formation of 8-oxo-dG. However, the formation of piperidine-labile lesions specifically at 5′-GG-3′ sites is a signature of base damage resulting from electron transfer. Singlet oxygen, on the other hand, is nearly equally reactive with all guanines and is only limited by diffusion (*16*, *17*). Typically, an enhancement of guanine damage in D_2O is supportive of a singlet oxygen mechanism since the lifetime of singlet oxygen is significantly longer in D_2O (*30*, *31*).

Methylene blue is known to generate singlet oxygen (*8*) and is included in this study for comparison to guanine damage proceeding by a purely electron-transfer-based mechanism. Methylene blue causes nonselective direct cleavage with a very low efficiency. Various results have been obtained by others for methylene blue-induced direct cleavage (*43*−*46*). Our results are in agreement with Friedman and Brown (*43*) but contrast with reports of direct cleavage at guanines with modified 3′-phosphates upon photolysis at 633 (*44*, *45*) and 420 nm (*46*). Irradiation of methylene blue at a variety of wavelengths does produce piperidine-sensitive lesions at each guanine. In agreement with previous reports (*47*, *48*), we find that guanine damage is enhanced in D_2O and oxygen, and 8-oxo-dG is detected as a major degradation product. Electron transfer between methylene blue and guanine or adenine has also been reported, which raises the possibility that the damage may result from a redox reaction with this intercalator. However, the kinetics for these photoinduced oxidations as well as the recombination reactions are extremely fast ($k \sim 10^{12}$ s^{-1}) (*49*) and may prohibit the trapping required for irreversible damage. The essentially equal cleavage at all guanine residues in this oligonucleotide indicates that a predominantly singlet oxygen-mediated pathway is operative for the damage of DNA by methylene blue.

In fact, oxidative DNA damage by electron transfer shows many of the same indicators as singlet oxygen damage. 8-Oxo-dG can be formed; O_2 is required, and depending on the photophysical properties of the intercalator, D_2O may enhance the damage. However, two factors are useful in confirming an electron-transfer-mediated pathway. First, 5′-GG-3′ specificity cannot be achieved by the diffusible species and is strongly indicative of an electronic vacancy which is afforded equilibration through the DNA base stack. Second, when synthetic methodologies are available, the investigation of guanine oxidation over long molecular distances can provide evidence for an electron-transfer mechanism. Since the radius of diffusion for singlet oxygen appears to be quite short (*12*), damage by this diffusible species is highly distance-dependent. Thus, electron-transfer reactions through the base stack, which have been shown to proceed with equal efficiencies for distal and proximal sites separated by 20 Å, can easily be identified using reactants covalently tethered to discrete sites in DNA duplexes. Long-range oxidation reactions proceeding by electron transfer, in contrast to those limited by diffusion or direct interactions, are afforded long migration distances by the extended π-stack of aromatic heterocycles within the DNA helix. Indeed, the DNA base pair stack now appears to mediate a broad range of highly efficient electron-transfer reactions (*10*−*15*, *28*).

The similarity in the long-range reactivity observed for tethered $Rh(phi)_2dmb^{3+}$ and ethidium indicates that facile hole migration through the DNA base stack may be a general phenomenon. This organic intercalator and metallointercalator have dramatically different photophysical properties, structural properties, and DNA binding characteristics. Ethidium binds DNA from the minor groove, while the Rh(III) intercalator binds from the major groove with exposed ancillary ligands residing in this groove. However, it appears that these structural details are not important in imparting long-range reactivity. Both species are stabilized by intercalative stacking in the base-paired DNA duplex. With this stacking, a pathway for charge transport through the array of stacked, coupled DNA base pairs is accessed, permitting charge migration and resultant oxidative damage over long molecular distances.

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