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Guanine Radical Cations Are Precursors of 7,8-Dihydro-8-oxo-2′-deoxyguanosine But Are Not Precursors of Immediate Strand Breaks in DNA

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Abstract: Biphotonic photoionization of frozen aqueous solutions of DNA at 248 nm has been shown by EPR spectroscopy to lead selectively to the guanine cation. In $H_2{}^{18}O$ under these conditions high levels of [^{18}O]-7,8-dihydro-8-oxo-2′-deoxyguanosine are produced in a dose-dependent manner, confirming direct formation of this oxidation product by hydration of the guanine cation. Photoionization of defined oligonucleotides did not give rise to significant levels of immediate strand breaks but generated G-specific alkali-labile sites that are readily cleaved by piperidine treatment. Authentic oligonucleotides containing 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG, **5**) sites are slowly cleaved at these sites on treatment with piperidine but at rates inconsistent with this being the source of the alkali-labile site. Photoionization of 7,8-dihydro-8-oxo-2′-deoxyguanosine-containing oligonucleotides demonstrated that this residue is highly susceptible to secondary oxidation and leads to formation of a markedly more alkali-labile lesion. Photoionization of 7,8-dihydro-8-oxo-2′-deoxyguanosine leads to release of 7,8-dihydro-8-oxoguanine suggesting that the alkali-labile site on photoionization of 7,8-dihydro-8-oxo-2′-deoxyguanosine-containing oligonucleotides is an apurinic site. However, the lack of significant 7,8-dihydro-8-oxoguanine release on photoionization of the parent oligonucleotides rules out secondary oxidation of 7,8-dihydro-8-oxo-2′-deoxyguanosine as the mechanism of formation of alkali-labile sites.

Introduction

Oxidative damage to DNA plays a key role in mutagenesis and carcinogenesis.[1,2] The focus of attention has been on reactive oxygen species such as hydroxyl and alkoxyl radicals, singlet oxygen, and superoxide ions, but equally important are single electron transfer reactions proceeding through radical cations. DNA damage by reactive oxygen species may lead to a number of modifications which in turn can be linked to subsequent tumor formation.[3] Following DNA exposure to reactive oxygen species over 20 base modifications have been identified, with the promutagenic lesion 8-oxodG being widely used as a marker for monitoring DNA damage.[4] 8-OxodG is formed in large amounts after exposure to reactive oxygen species and in certain tissues the increase in incidence has been correlated with subsequent tumor formation.[5] Given the importance of guanine oxidation in carcinogenesis it is important to characterize the

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mechanism of DNA damage. The use of site specifically modified DNA molecules provide models for probing both the chemical and biological effects of potential cancer promoting lesions.[6]

Ionizing radiation-induced damage to DNA as well as being important in its own right has provided a good model system in which to study redox damage to DNA generally, since the direct pathway for radiation damage to DNA occurs *via* formation of DNA base radical cations and radical anions. It is generally accepted that the site of lowest ionization potential in DNA is the guanine base,[7] leading to guanine radical cations. The site of electron addition in DNA is less clear cut; however, it is generally agreed to be at the pyrimidine bases.[8] The continuing interest in guanine oxidation products, particularly 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG, **5**), prompted this study to probe the decay pathway for the parent guanine cation using EPR spectroscopy and to determine whether direct strand breaks can arise from this initial radical cation. We report here that the guanine radical cation is not a precursor to direct strand breaks but gives rise to modified sites that lead to sequence specific cleavage at G sites on treatment with piperidine. We have shown that 7,8-dihydro-8-oxo-2′-deoxyguanosine is a major product from the guanine cation but is *not* the source of the alkali-labile sites. Furthermore secondary oxidation of 7,8-dihydro-8-oxo-2′-deoxyguanosine can occur but does not account for the observed alkali-labile sites.

Experimental Section

Laser Photolysis. DNA and oligonucleotide irradiations were carried out using a Lambda *Physic* excimer laser. The laser intensity at 248 nm was 8×10^{10} W m^{-2} with a pulse length of 20 ns and pulse repetition rate of 1 Hz. The DNA solutions (200 μL, 1 mg mL^{-1}) for dose-dependent product analysis studies using HPLC were irradiated in 1 mm pathlength quartz cuvettes at room temperature under oxic and anoxic conditions. Low temperature irradiations were also performed under liquid nitrogen in N_2 saturated 8 M $NaClO_4$ glasses. After irradiation the samples were annealed to room temperature, and the DNA was precipitated with 3 volume equiv of ethanol in order to remove the $NaClO_4$ for polyacrylamide gel electrophoresis (PAGE) and HPLC analyses.

EPR Analysis. The EPR methodology used in these experiments has been described previously.[8a,9] Briefly the EPR spectra were recorded at 77 K on a Jeol-RE1X X-band spectrophotometer interfaced with an Archimedes computer. Unless otherwise stated the spectra were recorded at a microwave power of 0.01 mW and a modulation amplitude of 0.2 mT.

Photoionization. The DNA radical cation was generated by photoionization of 25 mg mL^{-1} salmon testes DNA (Sigma) in transparent 8 M $NaClO_4$ glasses with 300 pulses of 248 nm UV light at 77 K. The perchlorate glasses suppress the formation of DNA radical anions and solvated electrons because they scavenge the photoejected electrons producing ClO_3^- and $O^{\bullet -}$.[5] Thus solute radical anion formation is suppressed in these glasses.

$$\mathrm{DNA} + \mathrm{ClO_4^-} + 2h\nu \rightarrow \mathrm{DNA^{\bullet +}} + \mathrm{ClO_3^-} + \mathrm{O^{\bullet -}} \qquad (1)$$

The EPR spectra were recorded directly at 77 K and after annealing using a copper block cryostat (Annealing refers to the warming of the sample to a prescribed temperature for 8 min followed by recooling to 77 K). The $O^{\bullet -}$ solvent signal is irreversibly lost on annealing the sample to 180 K or can be subtracted from the DNA spectrum by exploiting the differences in the spectra obtained at low and high microwave power.[9])

Radical Anions. The 8-oxodG radical anion was generated by γ-irradiation in 10 M LiCl glasses. This leads to the formation of $ClOH^{\bullet -}$ and $Cl_2^{\bullet -}$ electron loss centers and solvated electrons.[8] The electrons are mobile in these matrices and undergo addition to the solute even at 77 K to form the 8-oxodG radical anion. The radical anion spectrum was isolated by subtraction of the solvent signals from the 77 K spectrum and by annealing the sample to 155 K at which temperature the solvent radicals decay.

Determination of Sequence Specificity of Damage Induced by Photoionization. Two self-complementary oligonucleotides (A sequence 5′-ATATGCGCGCGCATAT (16mer) and B sequence 5′-GTGAGCATGCATGGGGAAAAGGGCCCTTTTCCCCATG-CATGCTCAC (46mer)) were synthesized (Applied Biosystems Model 394 DNA/RNA Synthesizer) on a 1 μmol scale and purified by HPLC[10] and denaturing polyacrylamide gel electrophoresis (PAGE),[11] respectively. The respective oligonucleotides (0.5OD units) were 5′ end labeled using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Gibco BRL).[12] Typically 10 pmol of ^{32}P- labeled oligonucleotide was dissolved in a 40 μL solution of 1 mg mL^{-1} salmon testes DNA (so as to match the DNA absorbance in the 8-oxodG analyses) in cylindrical quartz cuvettes with dimensions i.d. = 1.7 mm and o.d. = 4 mm. Following photoionization, strand break and alkali-labile lesions (30 min, 90 °C in 1 M piperidine) were assayed by 20% denaturing PAGE and quantified by phosphoimagery (Molecular Dynamics Laser Phosphoimagery System, Image Quant Version 3.3) and bands were assigned by reference to Maxam and Gilbert G lane markers.[13] The kinetics of strand break formation induced by the piperidine treatment of the photoionized oligonucleotide was assayed over a 4 h period for comparison with the alkaline degradation of 8-oxodG and the 46mer oligonucleotide with a single 8-oxodG (Glen Research, Sterling, VA 20166) substituted at position 16 from the 5′ end. The 8-oxodG-containing 46mer was purified by gel electrophoresis and its structure confirmed by Maxam−Gilbert sequencing.[13] The site of incorporation of the 8-oxodG followed from the automated synthesis but was also confirmed by piperidine treatment of the 8-oxodG-containing oligonucleotide without alkylation. A single cleavage band was seen on PAGE analysis that corresponded to position 16 from the 5′-end, and none of the remaining G sites showed any cleavage.

HPLC Analysis of 8-oxodG in DNA. Irradiated DNA (200 μL, 1 mg mL^{-1}) was converted to nucleosides by enzymatic hydrolysis using nuclease P_1, snake venom phosphodiesterase (Pharmacia), and alkaline phosphatase (Sigma).[14] Yields of 8-oxodG were quantified by HPLC with electrochemical detection (+0.6 V) using a Spherisorb ODS2 column and a mobile phase consisting of eluent A: 10% methanol in 50 mM KH_2PO_4 at pH 5.5 and eluent B: 50% methanol in 50 mM KH_2PO_4 at pH 5.5 at a flow rate of 1 mL min^{-1}. The gradient used was an isocratic elution of 100% A for 18 min followed by a linear gradient to 100% B at 21 min which was maintained for 6 min (T = 27 min) followed by a linear gradient back to 100% A at 30 min. Calibration of the 8-oxodG yield was performed using authentic 8-oxodG synthesized by the method of Kasai *et al.*[15] Detection of guanine (retention time 5.5 min) and an unidentified product (retention time 3 min) were performed at +0.9 V. Alkaline degradation of 8-oxodG was performed in 1 M aqueous piperidine at 90 °C. Sampled aliquots were diluted 10-fold with 50 mM aqueous KH_2PO_4 and analyzed by HPLC with UV and electrochemical detection.

^{18}O Experiment. DNA (0.1 mg; salmon testes; Sigma) in [^{18}O]-water (100 μL) was photoionized at 248 nm with 50 laser pulses. The photoionized DNA was degraded to the component bases by formic

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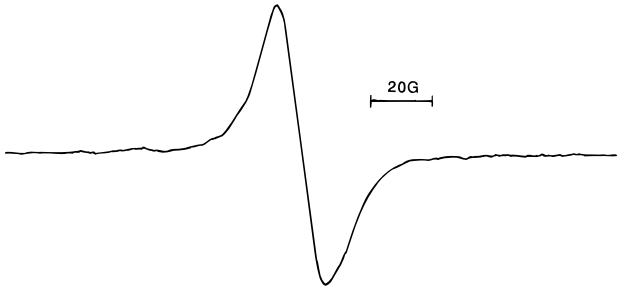

Figure 1. X-band EPR spectrum of the DNA radical cation generated by biphotonic photoionization of salmon testes DNA in a perchlorate glass at 77 K with a 248 nm KrF laser. The DNA electron loss center is assigned to the deprotonated guanine radical cation $G(-H^+)^{\bullet}$.

acid treatment, and the bases analyzed as their trimethylsilyl derivatives according to the literature procedure.[16] The GC-MS analysis of the 8-oxodG derivative was performed on a PE Autosystem GC interfaced to a PE Q-Mass 910 operating in selective ion monitoring mode, monitoring ions at 455 and 457. 8-OxodG derived from photoionizing DNA in unlabelled water gave a molecular ion of *m/z* 455.2 whereas the sample obtained for DNA solutions in [^{18}O]-water under otherwise identical conditions gave a *m/z* of 457.2 consistent with incorporation of a single oxygen from water.

Results and Discussion

Photoionization of Duplex DNA. Biphotonic photoionization of DNA has provided a convenient way of studying base radical cations in both DNA and its constituents.[9,17] Photoionization of DNA in frozen $NaClO_4$ glasses gives rise to a species that can be assigned with some confidence to the guanine base radical cation on the basis of its EPR spectrum, Figure 1. We have shown by EPR spectroscopy that photoionization of DNA and guanine-containing dinucleotides in perchlorate glasses at 77 K using a KrF excimer laser at 248 nm leads selectively to the guanine radical cation[18] implying that efficient hole-transfer must occur leading to the trapping of the most stable radical cation, and this has also been observed by others using a low intensity mercury lamp.[19] Under these conditions the perchlorate scavenges the electron (reaction 1), and we have shown that no base radical anion is formed,[18,20] and therefore provides a convenient system in which to study the chemistry of the cation component. Independent work by O'Neill *et al.*[21] irradiating at 193 nm and characterizing the transient species by UV spectroscopy has also confirmed the selective formation of guanine cation in mixed oligonucleotides at room temperature. These observations taken together provide convincing evidence that photoionizing mixed oligonucleotides gives rise to principally the guanine cation.

To determine whether the guanine radical-cation is a precursor of immediate strand breaks we have synthesized and purified ^{32}P-labeled oligodeoxynucleotides of defined sequence and photoionized them under ambient, oxic, and anoxic conditions both at room temperature and at 77 K and analyzed the products by denaturing polyacrylamide gel electrophoresis (PAGE), Figure 2 (parts a and b). The phosphoimaging gel-scans demonstrate that there are no significant immediate strand breaks formed for either the 16mer (Figure 2a lower trace) or the 46mer (Figure 2b lower trace), in contrast to the reports of Croke *et al.*[22] However, treatment with hot piperidine induces strand breaks showing marked base-specificity for G sites. The intensity of the bands present before piperidine treatment are <5% of those after piperidine treatment. The gel scans shown in Figure 2 are for experiments under ambient atmosphere at room temperature, but the same sequence selectivity and approximate level of damage was seen under all conditions, i.e. under ambient, O_2 and N_2 atmosphere, and photolyzing at room temperature and 77 K. The yields of alkali-labile sites as a percentage of total oligonucleotide concentration initially increases linearly with the fluence but reaches a plateau after a

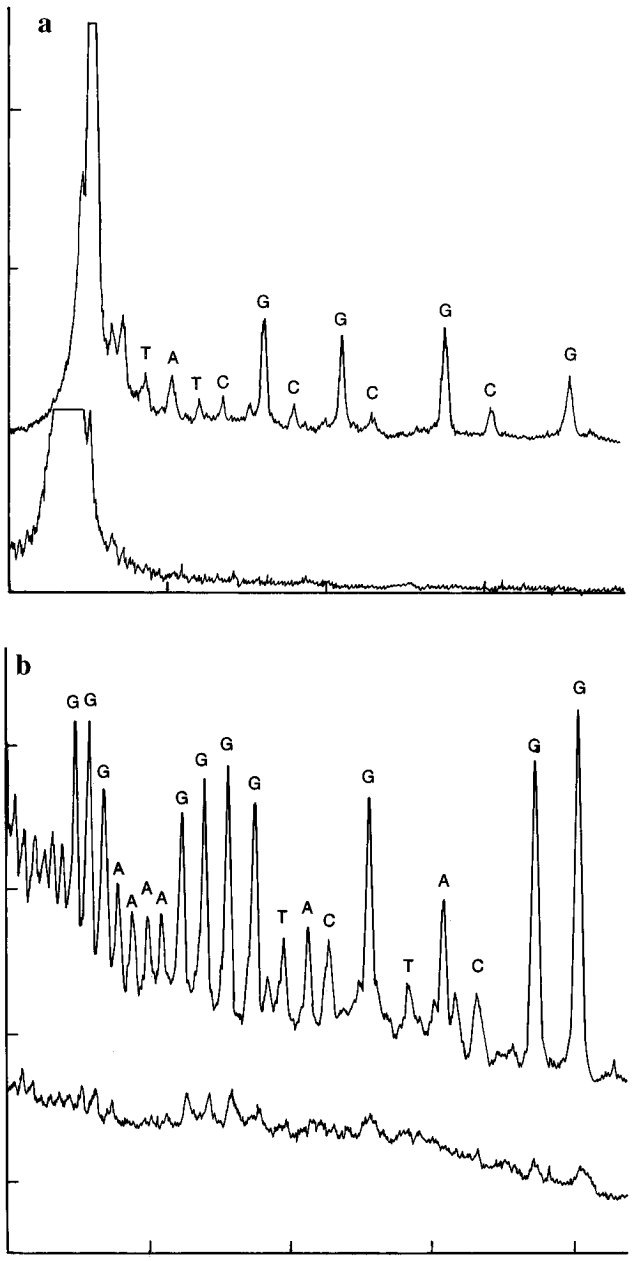

Figure 2. Phosphoimager gel scans of photoionized ^{32}P-labeled oligonucleotides showing no significant immediate strand breaks on photooxidation (lower plot) and extensive alkali induced strand breaks after treatment with hot piperidine (upper plot). Both of the experiments shown were conducted at room temperature and ambient atmosphere, but qualitatively the results were identical for irradiations performed at room temperature and 77 K and under N_2 and O_2: (a) Sequence (A) 5′ATATGCGCGCGCATAT and (b) Sequence (B) 5′GTGAGCATG-CATGGGGAAAAGGGCCCTTTTCCCCATGCATGCTCAC

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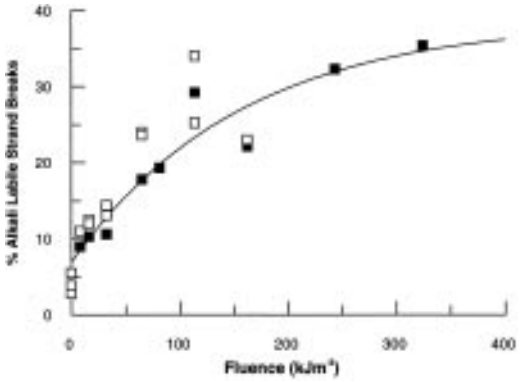

Figure 3. Dose dependent formation of alkali sensitive lesions (ASLs) in the 46 base oligonucleotide showing the fraction of alkali induced strand breaks as a function of fluence when photoionized at room temperature under N_2 (■) and ambient (□) conditions.

fluence of ca. 140 kJ m^{-2} (Figure 3). As can be seen from Figure 3, within the large experimental errors, there appears to be little difference between experiments under oxic and anoxic conditions. These results with defined oligonucleotides are in broad agreement with the results of Kovalsky *et al.*[23] using plasmid DNA. These authors also observed alkali-labile sites at pyrimidine residues when contiguous pyrimidines occur, and they explained this in terms of monophotonic processes involving pyrimidine cyclobutane dimer and pyrimidine-pyrimidone 6−4 photoproduct formation (although it is not clear that such modifications are expected to be particularly alkali sensitive). However, in Figure 2 (parts a and b) it can be seen that lower levels of cleavage occur at sites other than G and where two contiguous pyrimidines do not exist implying that some minor alternative radical pathways must exist. For example we have recently reported direct EPR spectroscopic support for a pathway from the cytosine radical cation to a C1′ sugar radical that could possibly contribute to this background.[9a] The observation that $G^{\bullet+}$ does not lead directly to a strand break is important since it has implications for damage to DNA induced by γ-irradiation under conditions of direct damage. Despite earlier assumptions it is now clear that $G^{\bullet+}$ generated by γ-irradiation under conditions of direct damage does not lead directly to strand breaks with significant efficiency.[24]

8-OxodG Formation from the Guanine Radical Cation. The nature of the alkali-labile site is intimately linked with the decay pathway for the guanine radical cation. In a recent study Kasai *et al.*[25] reported the formation of significant levels of 8-oxodG in the photolysis of DNA in the presence of the photosensitizer riboflavin, in a reaction presumed to proceed *via* the guanine radical cation. Our demonstration by EPR spectroscopy of the direct, selective formation of the guanine radical cation on biphotonic photoionization of DNA at 248 nm allowed us to examine this proposal. Although 8-oxodG has been observed on γ-irradiation under conditions favoring indirect damage, hitherto it has not been unambiguously demonstrated under direct damage conditions, i.e., *via* the guanine radical cation. Indeed, it has been inferred that 8-oxodG is *not* formed from the radical cation from studies on the nucleoside,[26] and this may reflect different competing reactions available to the guanine cation in nucleosides and mononucleotides as compared to the case in duplex DNA.

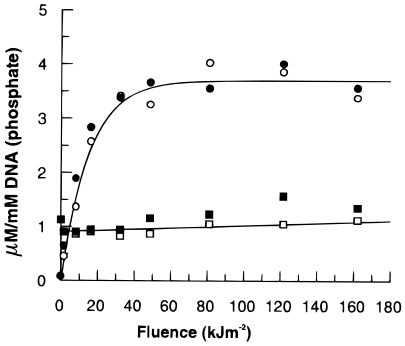

Figure 4. Dose dependent formation of 8-oxodG (*circles*), and guanine release (*squares*) in O_2 (full symbols, ● and ■) and N_2 (open symbols, ○ and □) saturated solutions of DNA. The products were measured by HPLC and electrochemical detection following enzymatic hydrolysis.

Photoionization of DNA at 248 nm led to the formation of 8-oxodG in quantities comparable to the levels of alkali-labile lesions at the G sites as shown by HPLC using electrochemical detection. The yield of 8-oxodG initially increased linearly with the number of laser pulses but subsequently reaches a plateau and begins to decline at a fluence of approximately 40 kJ m^{-2} which is a considerably lower dose than that required for saturation of the alkali-labile lesions, Figure 4. The yields of 8-oxodG were the same in oxygenated and deoxygenated systems (N_2 purging) ruling out singlet oxygen-mediated processes.[27] This observation contrasts with the study of Candeias and Steenken[26] on irradiation of the nucleoside at 193 nm who reported an *enhancement* of the formation of 8-oxodG in the presence of oxygen and with the study of Kasai *et al.*[25] who observed a *decrease* in the yield of 8-oxodG in the riboflavin photosensitized photoionization of DNA in the presence of oxygen. The latter effect may simply be due to the quenching of the triplet riboflavin by O_2.

This provided a good system in which to address the issue of whether hydration of the guanine cation can occur to give 8-oxodG in DNA. Photoionization of aqueous solutions of DNA at 248 nm has been shown to lead to formation of the guanine cation and, unlike 193 nm irradiation, ionization of water and other sites such as the sugar phosphates do not occur because of the lack of absorption of these species at this wavelength. Photoionizing DNA in $H_2{}^{18}O$ followed by degradation and derivatization of the base by literature procedures demonstrated that the 8-oxoguanine contained a single ^{18}O, confirming the observations of Kasai *et al.*[25] and providing compelling evidence for hydration of guanine radical cation as the first step in the pathway leading to 8-oxodG in oligonucleotides. A possible mechanism for conversion of guanine radical cations into 8-oxo-guanine is shown in Scheme 1. There are several elements to this scheme that are problematic. Firstly, EPR spectroscopy has not detected the 8-hydroxy-7,8-dihydroguan-7-yl radical (**3**) in γ-irradiation and photoionization of DNA and guanine nucleotides.[18,28] Secondly, it is not clear how the formal loss of the hydrogen atom would take place, although this may be overcome by deprotonation of the 8-hydroxy-7,8-dihydroguan-7-yl radical to give a radical anion followed by electron transfer. The immediate precursor to 8-oxodG would therefore be the corresponding radical anion (**4**).

The EPR spectrum of the π-radical anion of 8-oxodG formed by γ-irradiation of 8-oxodG in frozen aqueous LiCl glasses is shown in Figure 5. As expected the EPR spectrum is a narrow singlet with a line width of ca. 0.8 mT, spectral characteristics

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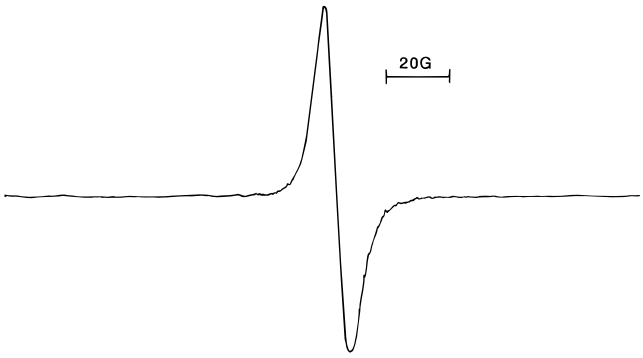
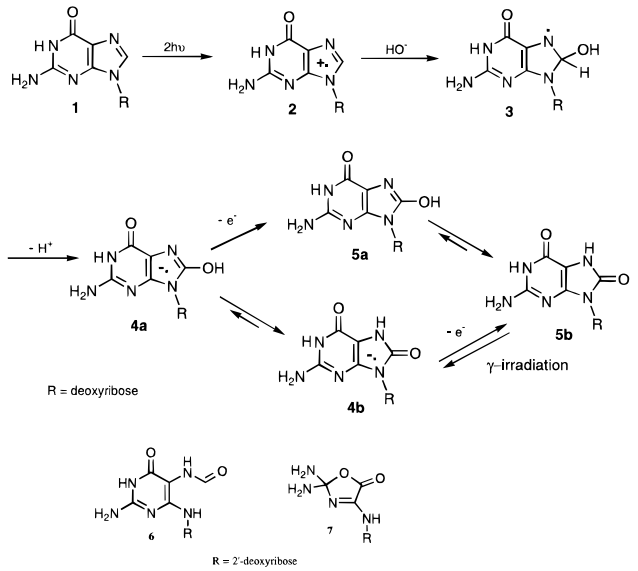


Figure 5. X-band EPR spectrum of the 8-oxodG radical anion generated by γ-irradiation in a 10 M LiCl glass at 77 K and annealing to 155 K to remove the background solvent signals. In contrast to the guanine radical anion the 8-oxodG radical anion does not C-8 protonate on annealing.

Scheme 1



similar to the radical *cation* derived from dG. Annealing of the 8-oxodG radical anion does not lead to any subsequent radical species indicating that, in sharp contrast to the deoxyguanosine radical anion, this radical shows no tendency to C-8 protonate in neutral glasses and is irreversibly lost on warming. It should be noted, however, that the π-radical anion formed from 8-oxodG by γ-irradiation in LiCl glasses is unlikely to be **4a** but its keto tautomer **4b**, whereas simple deprotonation of **3** will lead to the π-radical anion as the hydroxy tautomer **4a**. It is also not clear whether in the conversion of $G^{\bullet+}$ to 8-oxodG the tautomerization would precede or follow the electron transfer step. This suggests that the 8-hydroxy-7,8-dihydroguan-7-yl radical when formed will tend to deprotonate and must either mean that (**3**) is a relatively strong acid or that the equilibrium favors the radical anion because of the tautomerization that occurs after the deprotonation step. Alternatively, at low temperatures **4a** could be a relatively strong reducing species and electron transfer (for example to a pyrimidine) would be rapid. Preliminary *ab initio* calculations have shown that **4b** is more stable than **4a** by ca. 97 kJ mol^{-1}, which is even more in favor of the keto tautomer than for **5**, where **5b** is more stable than **5a** by ca. 53 kJ mol^{-1}. Finally, it is interesting to note that the EPR spectra of the guanine π-radical cation (**2**) and the 8-oxoguanine π-radical anion are both rather uninformative singlets such that thermal conversion of the former into the latter

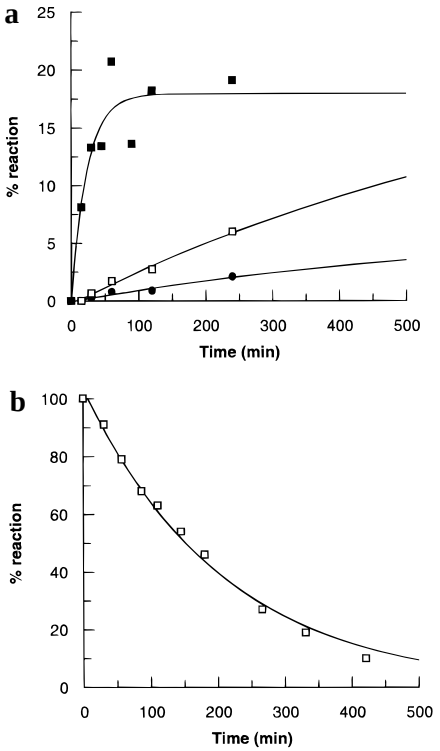


Figure 6. (a) The rate of piperidine-induced strand breaks in photoionized DNA (■; 20 pulses under N_2) and for the degradation of 8-oxodG-containing oligonucleotide in the absence (□) and presence (●) of β-mercaptoethanol in 1 M piperidine at 90 °C. (b) The piperidine-induced degradation of 8-oxodG ($t_{1/2}$ = 150 min) in 1 M piperidine at 90 °C.

would be difficult to detect in the composite EPR spectra arising from irradiation of DNA.

In our hands, the photoionization of the nucleoside, deoxyguanosine, at 248 nm, under both N_2 and O_2, also gave rise to 8-oxodG, with a dose dependence similar to that for 8-oxodG formation in DNA (data not shown). The difference between these observations and the report by others[26] may relate to the irradiation wavelength and the ease of secondary oxidation of the product 8-oxodG. Irradiation of deoxyguanosine at 193 nm may not allow significant build up of 8-oxodG because of competing photochemical decay.

Alkali-Lability of 8-oxodG Sites. Clearly, 8-oxodG is a major base lesion in DNA following biphotonic photoionization at 248 nm. The yields of 8-oxodG are comparable to the estimated yields of strand breaks arising from the alkali-labile sites. To address the issue of whether the alkali-labile lesion is in fact the 8-oxodG we have looked at the kinetics of piperidine induced degradation of 8-oxodG and of synthetic oligonucleotides containing a single authentic 8-oxodG site. It was confirmed that the 8-oxodG nucleoside is indeed susceptible to alkaline hydrolysis, and decay of the 8-oxodG on treatment with 1 M piperidine at 90 °C under ambient atmosphere, as monitored by HPLC, showed a half-life under these conditions of 150 min, Figure 6b. Although the products of this piperidine-induced degradation of the modified nucleoside have not been identified, it is clear that it does not involve release of 8-oxoguanine that would yield an apurinic alkali-labile site if it occurred in an oligonucleotide. These results are qualitatively similar to those of Chung *et al.*,[30] but we observe a significantly slower rate of degradation ($t_{1/2} \approx 150$ min) of 8-oxodG in hot piperidine. However, the susceptibility of 8-oxodG to oxidative

degradation may explain the faster loss of starting material seen by Chung *et al.*[30]

In terms of the alkali-lability of the photoionized parent oligonucleotide, efficient strand cleavage was observed with the typical Maxam−Gilbert condition (1 M piperidine, 90 °C, 30 min), Figure 6a, and was considerably more facile than the degradation of 8-oxodG, Figure 6b. Because of this difference in rate it is improbable that the majority of the alkali-labile sites are 8-oxo guanine-containing residues. To address this issue directly we synthesized the 46mer oligonucleotide sequence with a single 8-oxodG substituted at position 16 from the 5′-end by standard automated oligonucleotide synthesis (Applied Biosystems Model 394 DNA/RNA Synthesizer) using the commercially available phosphitylated 8-oxodG monomer (Glen Research, Sterling, VA). Both the 8-oxodG-containing sequence and the parent unmodified sequences were purified by gel electrophoresis and their structures confirmed by Maxam−Gilbert sequencing. The site of incorporation of 8-oxodG follows from the automated synthesis and is confirmed by the cleavage at this site seen on treatment with piperidine (see below). Treatment of the 8-oxodG-containing oligonucleotide with piperidine (1 M, 90 °C, ambient atmosphere, 30 min) gave rise to a small amount of cleavage at the 8-oxodG residue, and the rate of cleavage appeared even slower than that seen for the degradation of the nucleoside, with only ca. 6% cleavage after 240 min, Figure 6a. Furthermore this piperidine-induced cleavage is slowed down by the addition of β-mercaptoethanol, suggesting that this cleavage may be due to slow oxidation. This profile is markedly different to that for the rapid development of strand breaks on piperidine treatment of the photoionized parent oligonucleotide. From this data it is clear that the major, rapid alkali-labile sites are *not* -oxodG. Although Chung *et al.*[30] reached a different conclusion, their own data would support this conclusion since only a fraction of their 8-oxodG-containing oligonucleotide was cleaved using typical Maxam−Gilbert conditions.

Semiquantitative estimates suggest that the yields of alkali-labile sites and 8-oxodG residues are comparable, and these clearly must arise from competing reactions of the guanine radical cation. Our estimate that the yield of immediate strand breaks is less than 5% of the alkali-labile sites therefore means that less than ca. 2% of the guanine cation can be reacting to give an immediate strand cleavage. This is an upper limit, and it could be considerably lower than this since it assumes that all of the guanine cation is reacting to give either the alkali-labile site or 8-oxodG in equal yields. It has not been possible to determine independently the yield of the guanine cation.

8-OxodG is very susceptible to further oxidation and to determine whether the alkali-labile sites formed on photoionizing oligonucleotides could arise as a result of a secondary oxidation of the 8-oxodG lesions we have photoionized the 8-oxodG-containing oligonucleotide. As with the parent oligonucleotide no significant immediate strand breaks are formed. However, on piperidine treatment predominant cleavage occurred at the

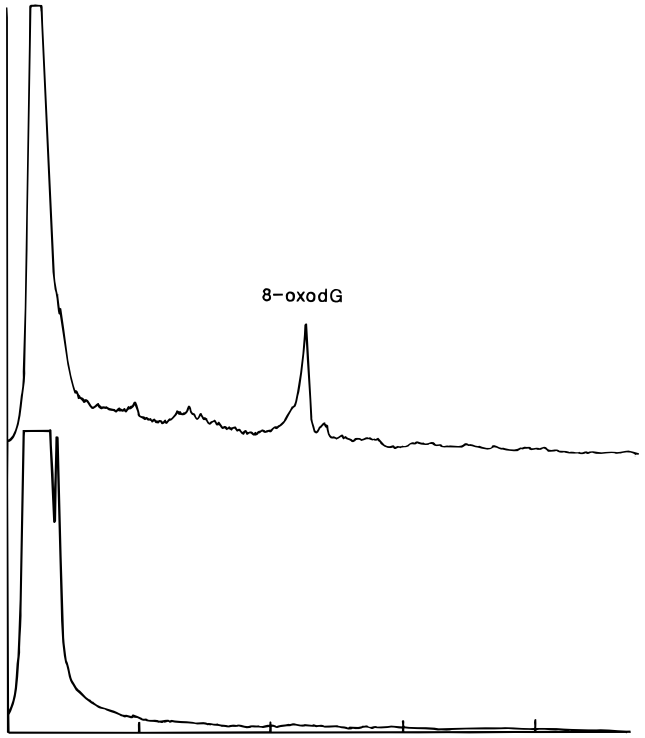


Figure 7. Phosphoimager gel scans of photoionized ^{32}P-labeled oligonucleotide (46mer) containing an authentic 8-oxodG modified base (X) at position 16 from the 5′ end photoionized under N_2 and O_2. The gel shows no significant immediate strand breaks on photooxidation (lower plot) and site specific strand breakage at 8-oxodG after treatment with hot piperidine (upper plot). The results with and without oxygen are quantitatively the same. 5′GTGAGCATGCATGGGXAAAAGGGC-CCTTTTCCCCATGCATGCTCAC.

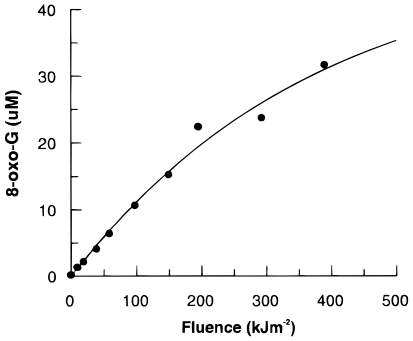
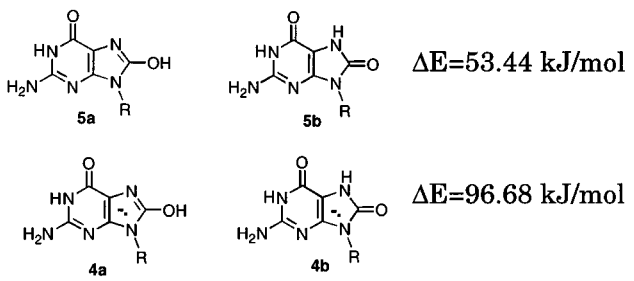


Figure 8. Dose dependent formation of 8-oxoguanine on photoionization of 8-oxodG under N_2. EPR spectroscopy revealed that photoionization of 8-oxodG generates a π-radical cation with a narrow singlet EPR spectrum. Presumably the release of 8-oxoguanine involves the hydrolytic cleavage of the glycosidic bond to form an alkali-labile apurinic site. However, insufficient 8-oxoguanine is released on photoionization of DNA for this secondary oxidation of 8-oxodG to account for the alkali-labile sites.

(29) Preliminary *ab initio* Hartree-Fock calculations (See: Gaussian 94 (Revision B.2); Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T. A.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L. ; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J. ; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. Gaussian, Inc.: Pittsburgh, PA, 1995.) using a 6-31G** basis set gave the relative energies shown below with the keto tautomer the more stable in each case. The difference in energy between 8-oxodG (**5b**) and the corresponding radical anion (**4b**) is estimated to be 110 kJ mol^{-1}.



(30) Chung, M.-H.; Kiyosawa, H.; Ohtsuka, E.; Nashimura, S.; Kasai, H. *Biochem. Biophys. Res. Comm.* **1992**, *188*, 1−7.

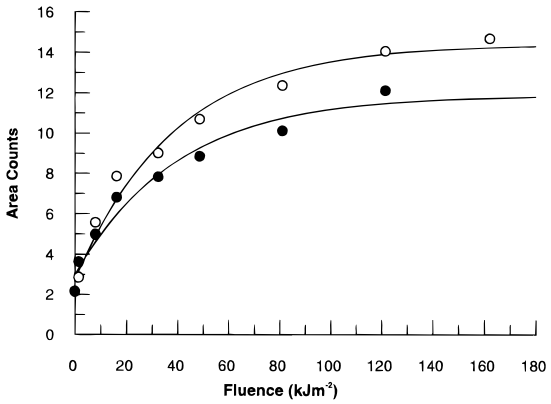


Figure 9. The dose dependent formation of an unidentified electrochemically active product on photoionization of DNA under N_2 (○) and O_2 (●) which may be associated with the alkali-labile site.

8-oxodG site (Figure 7) indicating that 8-oxoguanine is more readily (preferentially) photoionized than the guanine bases and gives rise to a lesion that is considerably more alkali-labile that the 8-oxodG site itself. We have shown that photoionization of 8-oxodG nucleoside itself leads to release of 8-oxoguanine, (Figure 8), but, since little of this product appears to be formed when DNA itself is photoionized, it seems unlikely that secondary oxidation of the 8-oxodG residues formed within the DNA could account for the alkali-labile site. Furthermore, the alkali-labile sites at G positions are evident at very low doses where the build up of 8-oxodG is still linear. It is worth commenting here that the failure to see significant yields of 8-oxodG by oxidation of guanosine nucleosides and mononucleotides using potent oxidising agents may reflect rapid further oxidation of the product 8-oxodG.[26]

Nature of the Alkali-Labile Sites. What alternative G-modifications could account for the alkali-labile lesion, having ruled out the involvement of 8-oxodG sites? Firstly, it is worth noting that we do not see any significant release of guanine (Figure 4) which might accompany formation of an apurinic site, a well characterized alkali-labile site. The very slight dose-dependent increase in guanine levels seen in the presence of O_2 could indicate a very minor pathway involving C1′ deprotonation that would lead to base release and the formation of 2-deoxyribono-1,4-lactone,[31] but the levels of guanine release are far too small to account for the bulk of the rapid alkali-labile sites. In terms of other major guanosine lesions that have been reported there would appear to be two significant possibilities, Fapy Gua (**6**) and the 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone (**7**). The oxazolone (**7**) recently identified by Cadet as a major radical oxidation product of guanine nucleosides and nucleotides seems the most likely since not only is it a major oxidation product of guanine but also it has been shown to be sufficiently alkali-labile to account for the facile piperidine cleavage of the photoionized oligonucleotides.[32] In the HPLC analysis of the products from photoionization of DNA, in addition to the 8-oxodG, we have observed the build up of a second significant electrochemically active product (HPLC retention time 3 min) with a dose dependence similar to that for the formation of the alkali-labile sites (Figure 9). This product is formed under both oxic and anoxic conditions, although there appears to be a slight decrease in levels in the presence of oxygen. This product has not yet been characterized, but interestingly Fapy Gua (**6**) has been shown to be electrochemically active[33] and therefore could represent a possible candidate. However, Fapy Gua formation should be favored under anoxic conditions and yet we see little change in the alkali labile sites comparing N_2 with ambient conditions. It is possible that the lack of pronounced oxygen effect may be due to difficulties in maintaining rigorously anoxic conditions.

Conclusions

In conclusion we have shown the selective formation of the guanine cation on photoionization of DNA and oligonucleotides and that this guanine cation is not capable of initiating significant levels of immediate strand breaks. We have provided perhaps the most definitive proof that the guanine cation is a precursor to 8-oxodG residues in oligonucleotides and DNA and that the mechanism of formation involves hydration of the guanine cation. These 8-oxodG sites are slowly cleaved in hot piperidine but at rates inconsistent with them being the source of G-specific alkali-labile sites. We have shown that oligonucleotides containing an authentic 8-oxodG site are readily and selectively further oxidized at this site to give a lesion presumed to be an apurinic site. The fact that 8-oxodG sites are detected at significant levels in genomic DNA provides an intriguing suggestion for the nature of radiation (and indeed general oxidation) "hot-spots" in DNA. On the basis of our observations, accumulation of higher levels of 8-oxodG would certainly sensitize DNA to oxidative damage.

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