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Oxidative Modification of Guanine Bases Initiated by Oxyl Radicals Derived From Photolysis of Azo Compounds

Jie Shao, Nicholas E. Geacintov, and Vladimir Shafirovich *

Chemistry Department, 31 Washington Place, New York University, New York, New York 10003-5180

Abstract

Oxidative damage to guanine bases initiated by photolysis of the water-soluble radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) has been investigated by laser kinetic spectroscopy. In the neutral oxygenated aqueous solutions, 355 nm laser flash photolysis of AAPH generates a whole spectrum of free radicals including 2-amidinoprop-2-peroxyl ($\text{ROO}^\bullet$), 2-amidinoprop-2-oxyl ($\text{RO}^\bullet$), and superoxide ($\text{O}_2^{\bullet-}$) radicals. These oxyl radicals with negligible absorption in a near UV – visible range were monitored in the reactions leading to the products with characteristic absorption spectra. This approach reveals that $\text{RO}^\bullet$ radicals induce fast one-electron oxidation of 2'-deoxyguanosine (dG) to form guanine neutral radicals, $\text{dG}(-\text{H})^\bullet$. In contrast, $\text{ROO}^\bullet$ radicals do not react with observable rates with dG. The $\text{O}_2^{\bullet-}$ radicals were detected using a classical test reaction with tetranitromethane to form nitroform. The major pathway for formation of the end products of guanine oxidation is combination of the $\text{G}(-\text{H})^\bullet$ and $\text{O}_2^{\bullet-}$ radicals to form 2,5-diamino-4*H*-imidazolone (Iz). This mechanism was confirmed by analysis of the end products produced by oxidation of two substrates: (1) guanosine derivative 2',3',5'-tri-*O*-acetylguanosine (tri-*O*-Ac-G), and (2) the 5'-d(CCATCGCTACC) sequence. The major products isolated by HPLC and identified by mass spectrometry methods were the tri-*O*-Ac-Iz and 5'-d(CCATC[Iz]CTACC) products.

Introduction

Persistent inflammation, which is a fundamental physiological defensive reaction against a variety of viral and microbial infections, environmental pollutants, tobacco smoke, and other exogenous factors, becomes harmful if transformed to a chronic form.¹⁻³ Oxidative stress associated with chronic inflammation is characterized by overproduction of reactive oxygen species (ROS) implicated in a permanent oxidative damage to diverse biomolecules.⁴⁻⁶ These ROS induce enhanced peroxidation of membrane lipids and the accumulation of lipid hydroperoxides that break down into reactive intermediates such as electrophilic aldehydes, epoxides,⁷⁻¹⁰ and alkylperoxyl and alkoxyl free radicals.¹¹⁻¹³ The chemistry and genotoxic effects of covalent DNA adducts generated by electrophilic aldehydes and epoxides derived from lipid peroxidation have been studied extensively.¹⁴⁻¹⁹ Although oxidative damage to DNA exposed to lipid oxyl radicals has been documented,²⁰⁻²⁶ the detailed mechanisms remain poorly understood due to the complexities associated with the interconversion, fragmentation and structural characterization of lipid peroxyl and oxyl radicals.

The thermal and photochemical decomposition of azo compounds is a classical approach to the controlled generation of carbon-centered radicals which, in the presence of oxygen, are

*To whom the correspondence should be addressed. Chemistry Department, 31 Washington Place, New York University, New York, New York 10003-5181. Tel.: (212) 998 8456; Fax: (212) 998 8421; vs5@nyu.edu.

rapidly converted to alkylperoxyl radicals at close to diffusion-controlled rates.²⁷ The water-soluble 2,2'-azobis(2-amidinopropane) dihydrochloride (known as ABAP²⁸ or AAPH²⁹) has been extensively used to initiate lipid peroxidation,³⁰⁻³² to explore effects of oxidative stress on cultured cells³³⁻³⁶ and signaling responses associated with inflammation and aging.³⁷ Ingold and co-workers have shown that the thermal decomposition of AAPH in oxygenated neutral solutions generate both strand breaks and base modifications of supercoiled DNA.²¹⁻²³ In turn, neutral and negatively-charged peroxyl radicals derived from the decomposition of 2-methyl-*N*-(2-hydroxyethyl)propionamide and 2,2'-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide], respectively, were at least two orders of magnitude less effective in generating DNA strand scission. Termini *et al.* have shown that the DNA damage patterns produced by the decomposition products of 2,2'-azobis[2-(2-imidazolin-2-yl)propane] in the presence of oxygen exhibit damage predominantly at guanine sites.^{38,39} This base selectivity is not surprising because guanine is the most easily oxidizable nucleic acid base by one-electron transfer mechanisms.⁴⁰ However, ESR studies have shown that the incubation of AAPH with spin traps produces alkoxyl radical spin adducts. It is therefore questionable whether AAPH is an explicit source of peroxyl radicals.⁴¹⁻⁴³

In this work we investigated the kinetics of guanine oxidation by free radicals generated by photolysis of AAPH in neutral aqueous solutions. Our kinetic laser flash photolysis experiments indicate that 2-amidinoprop-2-peroxyl radicals (ROO[•]) derived from the photolysis of AAPH in oxygenated solutions do not exhibit observable reactivities toward 2'-deoxyguanosine (dG) and instead transformed to the highly reactive 2-amidinoprop-2-oxyl radicals (RO[•]), and the superoxide radical anions (O₂^{•-}) by the bimolecular pathways (Scheme 1).

The one-electron oxidation of dG and SCN⁻ anions by RO[•] radicals were directly monitored by the formation of guanine neutral radicals,⁴⁴ dG(-H)[•], and dithiocyanate radicals,⁴⁵ (SCN)₂^{•-}. The presence of O₂^{•-} radicals was detected by using the classical test reaction of O₂^{•-} with tetranitromethane, C(NO₂)₄ that results in the formation of nitroform, C(NO₂)₃⁻.⁴⁶ The end products of guanine oxidation initiated by the photolysis of AAPH in oxygenated solutions were investigated by a combination of HPLC separation, LC-MS, and MALDI-TOF/MS analysis. In the case of the free nucleoside 2',3',5'-tri-*O*-acetylguanosine (tri-*O*-Ac-G), the major product found was the 2,5-diamino-4*H*-imidazolone derivative, (tri-*O*-Ac-Iz). Site-selective oxidation of guanine in the oligonucleotide 5'-d(CCATCGCTACC) was initiated by photolysis of AAPH, resulting in the generation of the same major product, Iz. The formation of Iz lesions is consistent with a mechanism based on the combination of the G(-H)[•] and O₂^{•-} radicals (Scheme 1).⁴⁷

Experimental Procedures

Materials

Analytical grade chemicals, HPLC grade organic solvents, and Milli-Q purified (ASTM type I) water were used throughout; 2,2'-azobis(2-amidinopropane) dihydrochloride and 2'-deoxyguanosine from Sigma-Aldrich (St. Louis, MO) were used as received. The oligonucleotides from Integrated DNA Technologies (Coralville, IA) were purified, and desalted using reversed-phase HPLC. The integrity of the oligonucleotides was confirmed by MALDI-TOF/MS analysis. Phosphate buffer solutions were tested for residual traces of transition metals and, if necessary, were treated with Chelex.⁴⁸ The stock solutions of tetranitromethane (Sigma-Aldrich, St. Louis, MO) were prepared daily. The 50–80 μL aliquots of C(NO₂)₄ were extracted 3–5 times with ~5 mL of distilled water in order to remove water-soluble impurities.⁴⁶ After extraction, the C(NO₂)₄ liquid (25–40 μL) was suspended in ~5 mL H₂O; the supernatant with a C(NO₂)₄ concentration⁴⁹ of ~8 mM was separated by centrifugation and diluted with an equal volume of water.

Laser Flash Photolysis

The transient absorption spectra and kinetics of free radical reactions were monitored directly using a fully-computerized kinetic spectrometer system (~ 7 ns response time) described elsewhere.⁵⁰ Briefly, two solutions (e.g., containing AAPH and dG) were forced by a small positive gas pressure (0.3 – 0.5 atm) into a mixer and then through a quartz micro flow cell (~ 100 μ L) at a flow rate of 6 – 8 mL/min. A polarization beam splitter cube was used to adjust the laser energy incident on the cell in the 20 – 2 mJ/cm²/pulse range, as measured by a thermoelectric bolometer. The solution flow rate was controlled by two solenoid valves to provide for a complete sample replacement between successive laser shots. Individual laser pulses were selected from the nanosecond pulse trains (10 Hz) of a 355 nm Nd: Yag laser by a computer-controlled electromechanical shutter. The transient absorbance was probed along a 1 cm optical path by light from a pulsed 75 W xenon arc lamp with its light beam oriented perpendicular to the laser beam. The signal was recorded by a Tektronix TDS 5052 oscilloscope operating in its high-resolution mode that typically allows for a suitable signal/noise ratio after a single laser shot. All experiments were performed at room temperature (23 ± 2 °C). The rate constants of the free radical reactions were typically determined by least squares fits of the appropriate kinetic equations to the transient absorption profiles obtained in five different experiments with five different samples.

Photochemical Oxidation of Guanine

The samples of 100 nmol dG, or 10 nmol oligonucleotide in 1 mL air-equilibrated 5 mM phosphate buffer solutions, pH 7.0 containing 20 mM AAPH were irradiated for fixed periods by a light beam of a 100 W xenon arc lamp reflected at 45° from a dielectric mirror to select the 340 – 390 nm spectral range for photolysis. Immediately after irradiation the samples were subjected to analysis by HPLC methods. The chemical structures of the Iz nucleosides and oligonucleotide adducts were confirmed by MS analysis of the isolated end-products and by comparisons with the authentic standards.

Mass spectrometry

LC-MS analysis of the photoproducts was performed with an Agilent 1100 Series capillary LC/MSD Ion Trap XCT mass spectrometer equipped with an electrospray ion source as described elsewhere.⁵¹ The MALDI-TOF mass spectra were recorded in the negative mode using a Bruker OmniFLEX instrument.⁴⁷

Results

Laser Flash Photolysis of AAPH

Short-lived intermediates generated by laser flash photolysis of AAPH in neutral buffer solutions (pH 7), and their fates were directly monitored by nanosecond laser kinetic spectroscopy. Photoexcitation of AAPH in neutral buffer solution (pH 7.0) by 355 nm laser pulses results in a prompt appearance of the broad absorption band near 420 nm (Figures 1A and 1B). This absorption band, observed in the both air-equilibrated (Figure 1A) and deoxygenated (Figure 1B) solutions, can be assigned to the AAPH triplet excited state, ³AAPH. Indeed, the triplet excited states of azoalkanes have an absorption band in the visible spectral range. For instance, the triplet excited state of 2,3-diazabicyclo[2.2.1]hept-2-ene in deoxygenated benzene shows a weak absorption band at 500 nm.⁵²

In deoxygenated solutions, the decay of ³AAPH is associated with the transformation of the parent absorption band of ³AAPH at 420 nm to a new more intense band at 400 nm within a microsecond time window (0 – 20 μ s), as shown in Figure 1A. These spectral changes with two isosbestic points at 300 and 265 nm clearly indicate that ³AAPH stoichiometrically

transforms to a new intermediate with an absorption maximum at 400 nm, which was assigned to the diazenyl radical.

The 2-amidinopropyl radicals ($\text{R}^\bullet$) radicals formed together with the diazenyl radicals ($\text{R}-\text{N}=\text{N}^\bullet$) in reaction 2 were not detected because molecular absorptivities of the alkyl radicals are typically negligible in the UV – VIS spectral range.²⁷ The rate constant of the ${}^3\text{AAPH}$ cleavage [$k_2 = (8.1 \pm 0.8) \times 10^4 \text{ s}^{-1}$] was calculated from the rise of the transient absorption at 400 nm (black curve, Figure 1C). The diazenyl radicals decay (black curve, Figure 1C) by bimolecular pathways with the observed second order rate constant $k_{3,4}/\epsilon = (5.7 \pm 0.6) \times 10^5 \text{ cm s}^{-1}$, where ϵ is the extinction coefficient of $\text{R}-\text{N}=\text{N}^\bullet$ at 400 nm.

Oxygen rapidly reacts with the intermediates produced by the photolysis of AAPH and a fast decay of the transient absorption band at 420 nm is observed in oxygen-containing solutions (Figure 1B) instead of the rise of the transient absorption at 400 nm detected in deoxygenated solutions (Figure 1A). In the O_2 -concentration range of 0.27 – 1.3 mM the absorption changes are mostly associated with the quenching of ${}^3\text{AAPH}$ by O_2 .

Indeed, under these conditions, reaction 5 dominates, because the pseudo-first-order rate constants, k_5' calculated from the kinetic profiles recorded at 400 nm in air-equilibrated (blue curve, $[\text{O}_2] = 0.27 \text{ mM}$) and O_2 -saturated (red curve, $[\text{O}_2] = 1.3 \text{ mM}$) solutions (Figure 2C) are greater than the rate constant of the ${}^3\text{AAPH}$ cleavage (reaction 2). The corresponding second-order rate constant, $k_5 = (2.3 \pm 0.5) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ calculated from the k_5' and $[\text{O}_2]$ values is typical for reactions of triplet excited states with oxygen.⁵³ The detailed mechanism of this complex reaction that can include a cascade of fast consecutive reactions to form $\text{ROO}^\bullet$ radicals remains unknown and requires a further refinement. The contribution of radical reactions with oxygen can be significant at low concentrations of O_2 only, where $k_5[\text{O}_2] < k_2$.

Typically, alkyl radicals react rapidly with oxygen (reaction 6) with rate constants of the order of $10^9 \text{ M}^{-1}\text{s}^{-1}$.²⁷ The rate constant of reaction 7 can be measured at concentrations of $[\text{O}_2] < 0.05 \text{ mM}$. However, these investigations were beyond the scope of this work, because all further experiments were done in air-equilibrated or O_2 -saturated solutions.

One-Electron Oxidation of 2'-Deoxyguanosine Triggered by Photolysis of AAPH

Reactive species generated by the photolysis of AAPH in the presence of oxygen induce one-electron oxidation of 2'-deoxyguanosine. The guanine neutral radicals, $\text{dG}(-\text{H})^\bullet$, which are the products of this reaction, were identified by the appearance of the characteristic narrow absorption band at 315 nm (Figure 2).^{44,54}

The kinetic profiles recorded at 315 nm are mostly associated with the rise of the $\text{dG}(-\text{H})^\bullet$ absorption and exhibit a characteristic S-like shape. The presence of oxygen is critical for the oxidation of dG, because $\text{dG}(-\text{H})^\bullet$ radicals were not detected in argon-purged solutions (black curve, inset in Figure 2). In turn, variations of oxygen concentrations in the range of 0.27 – 1.3 mM do not affect the kinetics of dG oxidation, and essentially the same kinetic curves of $\text{dG}(-\text{H})^\bullet$ formation are observed in air-equilibrated (blue curve) and O_2 -saturated (red curve) solutions. This is a clear indication that at $[\text{O}_2] = 0.27 - 1.3 \text{ mM}$, the reaction of oxygen with the AAPH photolysis products is not the rate-determining step of dG oxidation. Indeed, the formation of $\text{dG}(-\text{H})^\bullet$ radicals that occurs within the time interval of 0 – 200 μs (inset in Figure 2) is slower than the decay of the AAPH photolysis products in the absence of dG, which is complete within $< 5 \mu\text{s}$ (Figure 1B). These results suggest that the primary products of AAPH photolysis rapidly react with oxygen to form the 2-amidinoprop-2-peroxyl radicals (reactions 5 – 7). The latter radicals are involved in further, slower processes to form the reactive species that oxidize dG, and these processes are responsible for the S-like shape of the kinetic curves (inset in Figure 2). Our following experiments indicate that these processes also produce the reducing species identified as superoxide radical anions, in addition to the dG oxidizing species.

The Photolysis of AAPH Generates Superoxide Radicals

The formation of $\text{O}_2^{\bullet-}$ radicals initiated by the laser flash photolysis of AAPH in air-equilibrated solutions was monitored using the selective reduction of tetranitromethane, $\text{C}(\text{NO}_2)_4$ by $\text{O}_2^{\bullet-}$, to form the nitroform anion, $\text{C}(\text{NO}_2)_3^-$.⁴⁶ The latter can be easily detected because of its strong absorption band at 350 nm.⁴⁶ Using this classic assay,⁵⁵ we found that the photolysis of AAPH in air-equilibrated solutions indeed produces $\text{O}_2^{\bullet-}$ radicals (Figure 3). The presence of oxygen and AAPH is required for the formation of nitroform, because in Ar-purged solutions and in the absence of AAPH (black line, Figure 3), no nitroform was detected. In 1 mM $\text{C}(\text{NO}_2)_4$ solutions, the $\text{O}_2^{\bullet-}$ radicals should produce nitroform within 0.5 μs , because this reaction is very fast and occurs with a rate constant of $1.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$.⁴⁶ However, the buildup of the nitroform absorbance at 350 nm is observed in the range of 0 – 400 μs and the kinetic profile has a characteristic S-like shape (red line, Figure 3), which is also clearly observed in the formation of $\text{dG}(-\text{H})^\bullet$ radicals (inset in Figure 2). These results suggest that nitroform formation is controlled by the formation of $\text{O}_2^{\bullet-}$ radicals, because reduction of $\text{C}(\text{NO}_2)_4$ by $\text{O}_2^{\bullet-}$ radicals itself is extremely fast and this step is not rate-determining.

Kinetics of Guanine Oxidation

We further investigated the effects of laser pulse energy (E) and the concentrations of dG and AAPH on the yields of formation of $\text{dG}(-\text{H})^\bullet$ (Y_{GR}) in air-equilibrated solutions. The values of Y_{GR} calculated from the 315 nm transient absorbance of the $\text{dG}(-\text{H})^\bullet$ radical ($\epsilon_{315} = 7.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ⁵⁶) at 200 μs are summarized in Figure 4.

The values of Y_{GR} increase linearly with increasing laser energy (Figure 4A). This is clear evidence that the one-electron oxidation of dG is initiated by a *single photon absorption*

mechanism. At constant laser energy, the value of Y_{GR} monotonically increases with the concentration of AAPH (Figure 4B). Increasing the AAPH concentration enhances the amount of light absorbed, thus rising the concentrations of the reactive species that oxidize dG to form $dG(-H)^{\bullet}$ radicals. The values of Y_{GR} rapidly rise as a function of increasing concentration of dG to attain an apparent constant value of $\sim 6 \mu M$ at $[dG] > 0.3 \text{ mM}$ (Figure 4C); in this range of dG concentrations, the major fraction of reactive species is trapped by dG, and thus a further rise of $[dG]$ does not enhance Y_{GR} .

The yields of nitroform (Y_{NF}) generated by the photolysis of AAPH in air-equilibrated solutions containing $1 \text{ mM } C(NO_2)_4$ were calculated from the 350 nm absorption maximum of $C(NO_2)_3^-$ at $600 \mu s$ using $\epsilon_{350} = 14.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$.⁴⁶ At concentrations of AAPH less than 10 mM , the values of Y_{NF} are very close to the values of Y_{GR} (Figure 4B). However, at $[AAPH] > 10 \text{ mM}$, the values of Y_{NF} become less than the Y_{GR} values, and at $[AAPH] = 40 - 50 \text{ mM}$ the values of Y_{GR} are greater than the Y_{NF} values by a factor of ~ 2.5 .

After an initial $20 - 25 \mu s$ rise of the $dG(-H)^{\bullet}$ absorbance at 315 nm can be described by pseudo-first-order kinetics with the rate constant, k_{GR} . The effects of laser pulse energy, and the concentrations of dG and AAPH on the k_{GR} values are summarized in Figure 5.

We found that the values of k_{GR} rapidly grow with increasing dG concentration and at $[dG] > 0.1 \text{ mM}$ attain the apparent constant value of $\sim 2 \times 10^4 \text{ s}^{-1}$ (Figure 5A). These results suggest that at $[dG] > 0.1 \text{ mM}$, the oxidation of dG is not the rate-determining step, and the lower limit of the rate constant of dG oxidation is greater than $2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$. In turn, a monotonic growth of the values of k_{GR} with increasing laser pulse energy (Figure 5B) or concentration of AAPH (Figure 5C) is observed.

The nitroform formation monitored at 350 nm (Figure 3) can be also described by pseudo-first-order kinetics with the rate constant, k_{NF} after an initial $30 - 50 \mu s$ (Figure 3). The values of k_{NF} for nitroform formation are close to the values of k_{GR} for generation of $dG(-H)^{\bullet}$ radicals at $[AAPH] < 10 \text{ mM}$ (Figure 4B). The difference between k_{GR} and k_{NF} increases with the growth of the AAPH concentrations, and at $[AAPH] = 40 - 50 \text{ mM}$ the values of k_{NF} are less than the k_{GR} values by a factor of ~ 4 .

To explain the observed changes in the k_{GR} and k_{NF} values associated with variations of the laser pulse energy and AAPH concentrations, which simply control the concentrations of the primary photolysis products (Figures 4A and 4B), we hypothesize that the reactive species oxidizing dG and reducing tetranitromethane are produced via decomposition of the common precursor. Analysis of the literature has shown that the potential intermediate, which can produce both $ROO^{\bullet}$ and $O_2^{\bullet-}$ radicals, is tetraoxide, $ROOOOR$.⁵⁵ The latter is formed via recombination of two 2-amidinoprop-2-peroxyl radicals (reaction 8) produced by photolysis of AAPH in the presence of oxygen (reactions 5 – 7) and then decomposes to form 2-amidinoprop-2-oxyl (reaction 9) and superoxide (reaction 10) radicals, among other decomposition products:

where x and y are the yields of $\text{RO}^\bullet$ and $\text{O}_2^{\bullet-}$ radicals in reactions 9 and 10, respectively. According to this mechanism, the oxidation of dG (reaction 11) and the reduction of tetranitromethane (reaction 12) are not rate-limiting and are controlled by the formation of $\text{RO}^\bullet$ and $\text{O}_2^{\bullet-}$ radicals. The rate-limiting step of this process (reactions 8 – 10) is determined by the transient concentrations of $\text{ROO}^\bullet$ radicals that are rapidly formed within $< 5 \mu\text{s}$ after an actinic laser flash (reactions 5 – 7). In the limit of low concentrations of $\text{ROO}^\bullet$ radicals (low laser energies or low AAPH concentrations), the rate-determining step is the recombination of two $\text{ROO}^\bullet$ radicals (reaction 8) that suggests a linear relationship between the k_{GR} and k_{NF} values and concentrations of $\text{ROO}^\bullet$ radicals ($k_{\text{GR}} \sim 2xk_8[\text{ROO}^\bullet]$ and $k_{\text{NF}} \sim yk_8[\text{ROO}^\bullet]$). In this limit a linear decrease of k_{GR} and k_{NF} with decreasing laser energy and AAPH concentrations, which control the yields of $\text{ROO}^\bullet$ radicals in reactions 5 – 7, is expected in agreement with our observations (Figures 5B and 5C). Using the data from Figures 4 and 5 we estimate the value of $k_8 \sim 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$; in comparison, the methylperoxyl radicals recombine with the rate constant of $3.7 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$.⁵⁷ In the limit of high concentrations of $\text{ROO}^\bullet$ radicals (high laser energies or high AAPH concentrations), the rate-determining step is the decomposition of the tetraoxide (reactions 9 and 10). The values of k_{GR} and k_{NF} do not depend on the concentrations of $\text{ROO}^\bullet$ radicals ($k_{\text{GR}} \sim 2xk_9$ and $k_{\text{NF}} \sim yk_9$). In this limit of high laser energies and high concentrations of AAPH, k_{GR} and k_{NF} approach constant values (Figures 5B and 5C) that yield an estimate of the ROOOOR lifetime of 15 – 20 μs .

The Reactive Species that Oxidize dG are not Hydroxyl Radicals

The species generated by AAPH photolysis in the presence of oxygen are highly reactive and rapidly oxidize dG to form $\text{dG}(-\text{H})^\bullet$ radicals. Since, these species have no characteristic absorption spectra in the spectral range that is convenient for the recording such spectra ($\lambda > 300 \text{ nm}$, inset in Figure 2), their reactivities can be probed by detecting the products derived from the oxidation of the appropriate electron donors. An example of this reaction is the one-electron oxidation of SCN^- anions to form $\text{SCN}^\bullet$ radicals, which in the presence of an excess of SCN^- anions exist in the dimeric form, $(\text{SCN})_2^{\bullet-}$.⁴⁵

The values of $E^\circ(\text{SCN}^\bullet/\text{SCN}^-)$ and $E^\circ((\text{SCN})_2^{\bullet-}/2\text{SCN}^-)$ are equal to 1.63 and 1.32 V vs NHE,⁵⁸ respectively, and the latter is very close to the value of $E_7 = 1.29 \text{ V}$ vs NHE for $\text{dG}(-\text{H})^\bullet$ radicals.⁴⁰ Figure 6 shows that the photolysis of AAPH in air-equilibrated solutions generates $(\text{SCN})_2^{\bullet-}$ radicals that are identified by the appearance of a characteristic transient absorption band at 472 nm.⁴⁵

The buildup of the $(\text{SCN})_2^{\bullet-}$ absorption band monitored at 472 nm has a characteristic S-like shape (Figure 6) that is also observed in the case of the $\text{G}(-\text{H})^\bullet$ (Figure 3) and nitroform (Figure 4) kinetics. The $(\text{SCN})_2^{\bullet-}$ radicals decay on the millisecond time scale (Figure 6) with the rate constant, $2k_{15}/\epsilon = 2.7 \times 10^5 \text{ cm s}^{-1}$; the value $\epsilon_{472} = 7.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ⁴⁵ yields the rate constant of radical recombination $k_{15} = (1.0 \pm 0.1) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.

The latter value is consistent with the literature value of $1.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ that was obtained at the higher ion strength of 0.2 M.⁴⁵

The oxidation of SCN^- anions to form $(\text{SCN})_2^{\bullet-}$ radicals by hydroxyl radicals has been routinely used for chemical dosimetry purposes in pulse radiolysis.⁴⁵ Redmond and co-workers used this reaction for the detection of $^\bullet\text{OH}$ radicals that are formed as a result of the laser flash photolysis of *N*-hydroxy-2(1*H*)-pyridone and *N*-hydroxypyridine-2(1*H*)-thione.⁵⁹ In the photolysis of AAPH, the formation of $^\bullet\text{OH}$ radicals can be excluded. We found that the addition of typical scavengers of $^\bullet\text{OH}$ radicals such as methanol that rapidly react with $^\bullet\text{OH}$ radicals ($k = 9.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ⁶⁰), does not affect the yield of $\text{dG}(-\text{H})^\bullet$ radicals at $[\text{CH}_3\text{OH}] = 10 \text{ mM}$, and at $[\text{CH}_3\text{OH}] = 100 \text{ mM}$ the value of Y_{GR} is $\sim 80\%$ of the that measured in the absence of methanol. The weak effect of the presence of such a typical $^\bullet\text{OH}$ radical scavenger on the formation of $\text{dG}(-\text{H})^\bullet$ radicals excludes the participation of $^\bullet\text{OH}$ radicals in the oxidation of dG.

End-Products of Guanine Oxidation Initiated by the Photolysis of AAPH

The laser flash photolysis experiments have shown that the photolysis of AAPH in the presence of oxygen generates highly oxidizing species, which mediate the fast one-electron oxidation of dG (Figure 3) and SCN^- anions (Figure 6). The reduction of superoxide radicals detected by the classical reaction with tetranitromethane implicates $\text{O}_2^{\bullet-}$ as an intermediate (Figure 4). According to our previous experiments, the $\text{G}(-\text{H})^\bullet$ and $\text{O}_2^{\bullet-}$ radicals rapidly combine with the rate constant of $4.7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ to form the unstable hydroperoxide, 5-HOO-G(-H) (reaction 16).⁴⁷

The latter rapidly decomposes with the formation of 2,5-diamino-4*H*-imidazolone that slowly hydrolyzes (half-life⁶¹ of $\sim 2.5 \text{ h}$ at 37°C) to the more stable 2,2,4-triamino-5-(2*H*)-oxazolone (Oz) as shown in Scheme 1.^{47,51} The parallel dismutation of $\text{O}_2^{\bullet-}$ radicals (reactions 17) occurs at pH 7 with the rate constant of $5.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$,⁶²

and cannot compete with reaction 16, which becomes the major pathway for the formation of stable end-products. Therefore, reaction pathways involving the formation of hydroxyl radicals derived from H_2O_2 are unlikely to contribute significantly to the oxidation of dG. Here, we explored the end products produced by the oxidation of two substrates: (1) the guanosine derivative 2',3',5'-tri-*O*-acetylguanosine, and (2) the guanine residue in the 5'-d (CCATCGCTACC) sequence used in our previous experiments.^{47,51}

We found that the photolysis of AAPH in air-equilibrated solutions containing tri-*O*-Ac-G initiates the efficient formation of tri-*O*-Ac-Iz derivatives isolated by reversed-phase HPLC and identified by LC-MS methods (Figure 7A).

Formation of tri-*O*-Ac-Iz occurs in a time-dependent manner (Figure 7B). The reaction products were also analyzed by HPLC-amperometric methods^{51,63} to determine if tri-*O*-Ac-8-oxoG was also formed. The tri-*O*-Ac-8-oxoG attained a level of 0.01% after an irradiation time of ~ 5 – 10 s (Figure 7B). Although this level is small as compared to the yield of tri-*O*-Ac-Iz, it is 3 – 5 times greater than the background levels in the unirradiated tri-*O*-Ac-G that served as a control for the photo-oxidation experiments. These results suggest that tri-*O*-Ac-8-oxoG is also formed but in minor quantities, in agreement with previous results.⁵¹

The photolysis of AAPH in air-equilibrated buffer solutions initiates the selective-oxidation of guanine bases in the single-stranded oligonucleotide, 5'-d(CCATCGCTACC). The major end product of guanine oxidation is the Iz lesion as in the case of the free nucleoside. The 5'-d(CCATC[Iz]CTACC) adduct was isolated by reversed-phase HPLC (Figure 8) and identified by MALDI-TOF/MS methods as described elsewhere.^{47,64}

The time-dependent increase in the yields of the 5'-d(CCATC[Iz]TACC) product (Figure 8B) is associated with the formation of minor quantities of 8-oxoG lesions. The latter were excised from the irradiated oligonucleotides by enzymatic digestions with nuclease P1 and alkaline phosphatase to the nucleoside level, and detected by the HPLC-amperometric methods. We found that the 8-oxoG lesions attained a level of 0.02% after a reaction time of 15 – 20 s. The addition of micromolar concentrations of Cu,Zn superoxide dismutase, Cu,Zn-SOD that induces the extremely fast catalytic dismutation⁶⁵ of $O_2^{\bullet-}$ radicals to O_2 and H_2O_2 suppresses the formation of Iz adducts (Figure 8C). The pronounced effect of Cu,Zn-SOD on the formation of the Iz lesions is a clear indication that Cu,Zn-SOD scavenges one of the reaction partners, the $O_2^{\bullet-}$ radical, and thus prevents the formation of the Iz lesions.⁴⁷

Discussion

The direct spectroscopic time-resolved measurements described here demonstrate that 2-amidinoprop-2-peroxyl radicals produced by the photolysis of AAPH in oxygenated solutions do not exhibit any observable reactivity with 2'-deoxyguanosine. The $ROO^{\bullet}$ radical is a mild oxidant that cannot efficiently mediate an electron abstraction from guanine. Indeed, the E_7 values of 1.0 – 1.1 V vs. NHE estimated for alkylperoxyl radicals⁶⁶ are not sufficient for a one-electron oxidation of dG ($E_7 = 1.29$ V vs. NHE).⁴⁰ In turn, the bond dissociation energies of the $ROO-H$ bonds are ~ 88 kcal/mol, regardless of the exact hydroperoxide structure.⁶⁷ This value is lower than the BDE of the weakest N1-H bond of guanine (94.3 ± 0.5 kcal/mol).⁶⁸ Even within the uncertainties in the BDE values, it is unlikely that fast H-atom abstraction from guanine residues by $ROO^{\bullet}$ radicals can occur. Hence, the low reactivity of $ROO^{\bullet}$ radicals with dG via a one-electron oxidation mechanism confirmed by direct laser flash photolysis experiments is most likely associated with the thermodynamics of the electron transfer/H-atom abstraction reactions.

One-electron oxidation of 2'-deoxyguanosine initiated by photolysis of AAPH detected by direct spectroscopic monitoring of the guanine neutral radicals (Figure 3) requires activation of the 2-amidinoprop-2-peroxyl radicals that occurs by the bimolecular pathway (reaction 8). The potential intermediate of this reaction is tetraoxide $ROOOOR$ arising from the combination of two $ROO^{\bullet}$ radicals, which decompose to form 2-amidinoprop-2-oxyl, $RO^{\bullet}$ (reaction 9) and superoxide, $O_2^{\bullet-}$ (reaction 10) radicals.⁵⁵ The electrophilic $RO^{\bullet}$ radicals with $E_7 = 1.55 - 1.65$ V vs NHE are stronger oxidants than the $ROO^{\bullet}$ radicals.⁶⁹ In agreement with thermodynamic predictions, the $RO^{\bullet}$ radicals rapidly oxidize dG ($E_7 = 1.29$ V vs. NHE)⁴⁰ and SCN^- anions

$[E^\circ((\text{SCN}^{\bullet-})_2/2\text{SCN}^-) = 1.32 \text{ V vs NHE}]$.⁵⁸ This is confirmed by the formation of the one-electron oxidation products, $\text{G}(-\text{H})^\bullet$ (reaction 11) and $(\text{SCN})_2^{\bullet-}$ (reactions 13 and 14) radicals that was directly monitored by laser flash photolysis (Figures 3 and 6). The detection of the 2-amidinoprop-2-oxyl radical spin adducts by the ESR methods provides an alternative confirmation for the formation of $\text{RO}^\bullet$ radicals initiated by thermal^{41,42} and photochemical⁴³ decomposition of AAPH in oxygenated aqueous solutions.

The superoxide radical derived from the cleavage of ROOOOR (reaction 10) is an anion form of the hydroxyperoxyl radical, $\text{HO}_2^\bullet$ ($\text{p}K_a = 4.8$).⁶² In contrast to organic peroxy radicals, which have only oxidizing properties, the $\text{O}_2^{\bullet-}$ radicals can both be reduced to hydrogen peroxide and oxidized to form O_2 . Hence, $\text{O}_2^{\bullet-}$ is the only peroxy radical which can rapidly reduce tetranitromethane (reaction 11).⁵⁵ Using this classic assay we were able to monitor the formation of $\text{O}_2^{\bullet-}$ radicals initiated by the photolysis of AAPH (Figure 4).

The combination of $\text{G}(-\text{H})^\bullet$ and $\text{O}_2^{\bullet-}$ radicals to form 2,5-diamino-4*H*-imidazolone (Figure 1) is the major pathway for the formation of the end products of guanine oxidation.⁴⁷ Here, this mechanism was confirmed by analysis of the end products produced by the oxidation of two substrates: (1) the guanosine derivative 2',3',5'-tri-*O*-acetylguanosine, and (2) the 5'-d (CCATCGCTACC) sequence. Indeed, the major products isolated by HPLC and identified by LC-MS/MS and MALDI-TOF/MS methods were the Iz derivative (Figure 7) and the 5'-d (CCATC[Iz]CTACC oligonucleotide adduct (Figure 8A). Suppression of the Iz lesion formation by the addition of micromolar concentrations of Cu,Zn superoxide dismutase (Figure 8B) provides a clear support that $\text{O}_2^{\bullet-}$ radicals participate in the formation of the Iz end products.⁴⁷

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Figure 1.

Laser flash photolysis of AAPH (30 mM) in deoxygenated, air-equilibrated and O_2 saturated buffer solutions (pH 7.0). Transient absorption spectra (Panels A and B) were recorded at the indicated delay times. Kinetic traces (Panel C) were monitored at 400 nm after a 355 nm single laser shot ($E = 20$ mJ/pulse/cm²).

Figure 2.

One-electron oxidation of dG induced by laser flash photolysis of AAPH in air-equilibrated buffer solutions (pH 7.0). The transient absorption spectra were recorded at the indicated delay times after a 355 nm single laser pulse excitation ($E = 20 \text{ mJ/pulse/cm}^2$) of 30 mM AAPH and 0.5 mM dG. The inset shows the kinetic traces due to dG(-H)• radicals at 315 nm in air-equilibrated (blue curve, $[O_2] = 0.27 \text{ mM}$), O_2 -saturated (red curve, $[O_2] = 1.3 \text{ mM}$), and deoxygenated (black curve) solutions.

Figure 3.

Kinetics of formation of $\text{O}_2^{\bullet-}$ radicals derived from the products generated by a 355 nm single laser pulse excitation ($E = 20 \text{ mJ/pulse/cm}^2$) of AAPH (30 mM) in air-equilibrated buffer solutions (pH 7.0). The progress of the reaction was monitored by the formation of nitroform at 350 nm derived from the reduction of $\text{C}(\text{NO}_2)_4$ (1 mM) by $\text{O}_2^{\bullet-}$ radicals. The inset shows the nitroform spectrum recorded at 600 μs after the actinic laser short.

Figure 4.

Effects of laser pulse energy (E) and concentrations of dG and AAPH on the yields of $\text{dG}(-\text{H})^\bullet$ radicals (Y_{GR}). Panel A: $[\text{dG}] = 0.5$ mM, $[\text{AAPH}] = 60$ mM; Panel B: $[\text{dG}] = 0.5$ mM, no tetranitromethane, or $[\text{C}(\text{NO}_2)_4] = 1$ mM, no dG, $E = 20$ mJ/pulse/ cm^2 ; Panel C: $[\text{AAPH}] = 30$ mM, $E = 20.5$ mJ/pulse/ cm^2 .

Figure 5.

Effects of laser pulse energy (E) and concentrations of dG and AAPH on the rate constant of the dG(-H) $\cdot$ formation (k_{GR}) Panel A: [AAPH] = 30 mM, E = 20 mJ/pulse/cm²; Panel B: [AAPH] = 60 mM, [dG] = 0.5 mM; Panel C: [dG] = 0.5 mM, no tetranitromethane or [C(NO₂)₄] = 1 mM, no dG, E = 20 mJ/pulse/cm².

Figure 6.

Kinetics of formation and recombination of $(\text{SCN})_2^{\bullet-}$ radicals at 472 nm initiated by a 355 nm single laser pulse excitation ($E = 20 \text{ mJ/pulse/cm}^2$) of AAPH (30 mM) in air-equilibrated buffer solutions, pH 7. The inset shows the $(\text{SCN})_2^{\bullet-}$ spectrum recorded at $60 \mu\text{s}$ after the actinic laser shot.

Figure 7.

End-products derived from the oxidation of tri-*O*-Ac-G (0.1 mM) initiated by the photolysis of AAPH (5 mM) in air-equilibrated phosphate buffer solutions. The solutions were photolyzed using 350 – 390 nm steady-state irradiation ($\sim 100 \text{ mW/cm}^2$) from a 100 W Xe arc lamp for fixed periods of time. (A) Reversed-phase HPLC elution profile of a sample irradiated for 5 s. The HPLC elution conditions (detection of products at 260 nm) were: 5 – 40% gradient of acetonitrile in 20 mM ammonium acetate over 60 min. The tri-*O*-Ac-Iz elutes at 24.5 min, and the unmodified tri-*O*-Ac-G at 29.8 min. (B) Dependence of the Iz, 8-oxoG tri-*O*-Ac-nucleosides on irradiation time. The latter were detected by the HPLC-amperometric method. 51,63

Figure 8.

End-products derived from the oxidation of 5'-d(CCATCGTACC) (10 μM) initiated by the photolysis of AAPH (300 μM) in air-equilibrated phosphate buffer solutions (pH 7.0). The solutions were photolyzed using 350 – 390 nm steady-state irradiation ($\sim 100 \text{ mW/cm}^2$) from a 100 W Xe arc lamp for fixed periods of time. (A) Reversed-phase HPLC elution profile of a sample irradiated for 15 s. HPLC elution conditions (detection of products at 260 nm): 5 – 15% gradient of acetonitrile in 50 mM triethylammonium acetate (pH 7) over 60 min. The 5'-d(CCATC[Iz]TACC) adduct **2** elutes at 25.8 min, and the unmodified 5'-d(CCATCGTACC) sequence **1** elutes at 27.5 min. The inset shows the time-dependent yields of the 5'-d(CCATC[Iz]TACC) adducts. (B) Effect of Cu,Zn-SOD on the yields of the 5'-d(CCATC[Iz]TACC) adducts produced by the 15 s irradiation.

SCHEME 1. Oxidation of Guanine Initiated by Photolysis of AAPH in Oxygenated Solutions
 Recombination of peroxy radicals, ROO[•] derived from photolysis of AAPH generates tetraoxide ROOOOR that spontaneously decomposes to form highly reactive oxyl radicals (RO[•]) and superoxide radicals (O₂^{•-}) with the yields of *x* and *y*, respectively.