Mechanism of Site-Selective DNA Nicking by the Hydrodioxyl (Perhydroxyl) Radical[†]

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ABSTRACT: In previous studies, the ability of the hydrodioxyl (perhydroxyl) radical [HOO•, the conjugate acid of superoxide ($O_2^{\bullet-}$)] to "nick" DNA under biomimetic conditions was demonstrated, and a sequence selectivity was observed. A background level of nonspecific nicking also was noted. This paper provides support for 5′-hydrogen atom abstraction from the deoxyribose ring as the initial event in the sequence-selective nicking by $O_2^{\bullet-}$/HOO•. Two experiments support the proposed mechanism. First, using a defined sequence 5′-^{32}P-labeled restriction fragment as the DNA substrate, only free (unalkylated) 3′-phosphate is produced at the site of nicking. Second, using poly (dA)•poly (T) as the substrate, furfural is formed in the reaction from deoxyribose ring breakdown. Both results are consistent with 5′-hydrogen atom abstraction for initiation of the site-selective nicking. Hydrogen atom abstraction at other sites of the deoxyribose ring and/or base oxidation and loss followed by strand scission likely are responsible for the nonspecific nicking. The 5′-abstraction mechanism contrasts to those elicited by other O_2-derived and metal-associated oxidants, which may provide a biomarker for the reactivity of HOO• *in vivo*.

O_2-derived oxidants are intimately involved in human disease initiation and propagation (Cross *et al.*, 1987). The basis of the involvement of oxidants in disease is through reaction with cellular targets; for example, DNA base oxidations and concurrent or subsequent "nicking" (strand breaking) can lead to genomic mutations which potentially initiate disease processes (Marnett & Burcham, 1993). Various investigators have demonstrated that oxidant-generating systems nick DNA *in vitro* by a variety of mechanisms [reviewed in Stubbe and Kozarich (1987) and von Sonntag (1987)]. In recent years, our laboratory has focused on the development and implementation of methods for generation of the individual O_2-derived oxidants in the absence of metals that can confound the identity of the reactive species generated (Aikens & Dix, 1991, 1992; Hess & Dix, 1992; Dix & Aikens, 1993; Dix *et al.*, 1994). These methods are being used to evaluate the mechanisms by which the individual oxidants react with biomolecules, which should lead to the identification of metabolites that could serve as biomarkers for the activity of each oxidant *in vivo*.

A particular focus of our laboratory has been the reaction of the hydrodioxyl (perhydroxyl) radical (HOO•),[1] the conjugate acid of superoxide ($O_2^{\bullet-}$), with biomolecules. The reactivity of $O_2^{\bullet-}$/HOO• with DNA was demonstrated previously (van Hemman & Meuling, 1975; Lesko *et al.*, 1980; Brawn & Fridovich, 1981) but not mechanistically characterized. This reaction was recently evaluated with a defined sequence DNA restriction fragment, and a unique (and surprising) sequence selectivity was observed. This selectivity contrasted with those of nicking exhibited by hydroxyl radical (HO•)-generating systems[2] and peroxyl radicals (ROO•), both of which are essentially nonselective (Dix *et al.*, 1994) in the absence of being incorporated into a DNA recognition system. In this paper, the mechanism of the sequence selectivity is evaluated using two distinct, but complementary, methods that provide support for 5′-hydrogen atom abstraction being the initial mode of attack of HOO•.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes and T4 polynucleotide kinase were obtained from New England Biolabs (Beverly, MA). Ampicillin, cytochrome *c* (type III), and G-50 Sephadex were obtained from Sigma (St. Louis, MO). The NACS column packing was obtained from Bethesda Research Labs (Gaithersburg, MD). Acetaldehyde (AA) and 2-*tert*-butyl-4-hydroxyanisole (BHA) were obtained from Aldrich (Milwaukee, WI). Desferroxamine methanesulfonate (desferal) was obtained from Ciba-Geigy (Summit, NJ) and [γ-^{32}P]-ATP from Du Pont-New England Nuclear (Wilmington, DE). Boeheringer Mannheim (Indianapolis, IN) supplied xanthine oxidase (XO) and calf intestinal alkaline phosphatase. XL1-Blue bacteria and pBluescript SK (−) plasmid were obtained from Stratagene (La Jolla, CA). Distilled water was filtered through a Milli-Q (Millipore, Bedford, MA) water purifica-

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[1] Abbreviations: HOO•, hydrodioxyl (perhydroxyl) radical; $O_2^{\bullet-}$, superoxide; HO•, hydroxyl radical; ROO•, peroxyl radical; AA, acetaldehyde; BHA, 2-*tert*-butyl-4-hydroxyanisole; XO, xanthine oxidase; GC−MS, gas chromatography−mass spectrometry; bp, base pair; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; AAPH, 2,2′-azobis(amidino)propane; Cu/OP, copper/*o*-phenanthroline.

[2] The actual oxidant being generated in "HO•-generating systems" such as the Fenton reaction is controversial (Dix & Aikens, 1993), as metal−oxo complexes may be generated concurrently.

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tion system before use. All other solvents and chemicals were obtained at the highest available purity from standard chemical supply firms. All buffers were prepared from distilled, deionized water using purified buffer salts and were Chelex treated to remove trace contaminating metals, as previously described (Aikens & Dix, 1991; Dix & Aikens, 1993). Reaction vessels (microfuge tubes) and pipette tips were autoclaved prior to use.

Spectrophotometric measurements were made using a Perkin-Elmer Lambda 4A UV/vis spectrophotometer interfaced with an ESC personal computer. Data were acquired and analyzed using UVS Spectroscopy Software from Softways (Moreno Valley, CA). The temperature for UV time course studies was controlled to within ±1 °C by use of a Lauda Instruments thermostatted circulating water bath plumbed to the spectrophotometer. Either a Sorvall (RC2B; GS3) centrifuge or an Eppendorf microcentrifuge was used for precipitation. Gas chromatography−mass spectrometry (GC−MS) was performed on a Finnigan 4000 instrument with the carrier gas at 8.5 kPa, the injection port at 250 °C, and electron ionization at 70 eV. Samples were eluted through a 30 m 5% phenylmethylpolysiloxane (DB-5%) capillary column using a temperature gradient from 50 to 250 °C over 20 min.

Preparation of Plasmids and Radiolabeled DNA Fragments

XL1-Blue bacteria were grown overnight in LB medium supplemented with 100 μg/mL ampicillin. Bacteria were collected by centrifugation, and the supernatant was removed. Plasmid DNA [pBluescript SK (−)] was isolated from the cells using alkaline lysis (Sambrook *et al.*, 1989) and precipitated with two volumes of ethanol. The isolated DNA was collected by centrifugation and run on a 0.7% agarose gel to obtain only the supercoiled DNA (the farthest-eluting band), as significant amounts of nicked plasmid typically resulted from the purification. The agarose matrix containing supercoiled DNA was excised from the gel, and the plasmid was recovered after electroelution into 3 M sodium acetate and precipitation by 2 volumes of ethanol. The purified DNA was washed once with 70% ethanol and dried *in vacuo*.

Linear restriction fragments of defined size were made using modifications of procedures described by Hertzberg and Dervan (1984). The purified pBluescript SK plasmid was cleaved with *Bam*HI to produce a linear piece of DNA with 5′-overhang ends. The 5′-ends were dephosphorylated with calf intestinal alkaline phosphatase, and the DNA was precipitated with ethanol. The linear DNA was then treated with *Pvu*II to produce a 191 base pair (bp) fragment. The 191 bp fragment was purified on a 2% agarose gel and isolated by electroelution as described above. This procedure was repeated on the precipitated fragment to ensure purity. The purified fragment was then eluted with a minimal volume of solvent from a NACS desalting column and precipitated with ethanol. The 5′-end of the 191 bp fragment was labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. The labeled DNA was passed through two G-50 Sephadex columns and precipitated with ethanol. The 5′-^{32}P-end-labeled 191 bp fragment was then resuspended in water and used immediately.

Reactions with DNA Substrates

XO/AA Experiments. XO was dialyzed against 50 mM Tris-HCl buffer (pH 7.8) for 24 h and then further dialyzed against 50 mM Tris-HCl buffer (pH 7.8) containing 100 μM desferal for 24 h (Britigin *et al.*, 1990; Lloyd & Mason, 1990; Aikens & Dix, 1991). This removes exogenously bound metals that can catalyze Fenton chemistry. The concentration of the resulting enzyme solution was determined by a urate formation assay (Fridovich, 1970) run at 37 °C in a 50 mM Tris-HCl (pH 7.43)/20 μM desferal buffer containing 500 μM xanthine. For DNA-nicking experiments, AA was substituted for xanthine as the substrate. A cytochrome *c* assay (McCord & Fridovich, 1969; Aikens & Dix, 1991) was run at 37 °C with 20 mM AA as the substrate to determine the flux of $O_2^{\bullet-}$/HOO$^{\bullet}$ released from the enzyme; typically, full recovery of activity was observed after dialysis as assayed using either xanthine or AA as the substrate.

Reactions with the 5′-end-labeled 191 bp DNA fragment were run with a concentration of DNA of 6.6 μM (bp) that was ≥10^6 cpm in ^{32}P. Reaction mixtures also contained 50 mM Tris-HCl (pH 7.4), 47 μM desferal, and 20 mM AA unless otherwise noted. XO was added to give final concentrations as listed in the figure legends. All reaction mixrures were incubated for 2 h at 37 °C, and the DNA was ethanol precipitated and resuspended in loading buffer for polyacrylamide gel electrophoresis.

To determine the nature of the 3′-termini of the 5′-end-labeled 191 bp DNA fragment after the reaction with XO/AA, the pelleted DNA was resuspended in 50 mM Tris-maleate buffer (pH 5.9) containing 10 mM $MgCl_2$ and 0.3 M NaCl and heat denatured for 2 min at 90 °C (Povirk & Steighner, 1990). After the mixture cooled to room temperature, β-mercaptoethanol (10 mM final concentration) and 3 μL of T4 kinase (10 units/μL) were added, and the reaction mixtures were incubated at 37 °C for 4 h. The DNA was then ethanol precipitated and resupended in loading buffer for analysis by polyacrylamide gel electrophoresis.

Reactions with poly (dA)·poly (T) as the substrate were run in a manner identical to that of the experiments described above except that the homooligonucleotide duplex was substituted for the 191 bp DNA fragment. After reaction, the samples were heated to 90 °C for 2 h, cooled, and extracted three times with ethyl acetate. Following drying ($MgSO_4$), the organic layer was removed *in vacuo* and the residue redissolved in a minimal volume of ethyl acetate for GC−MS analysis as described above.

Maxam−Gilbert Reactions. Maxam−Gilbert sequencing reactions were run using the 5′-^{32}P-end-labeled 191 bp DNA fragment using literature protocols (Maxam & Gilbert, 1980; Sambrook *et al.*, 1989).

Polyacrylamide Gel Electrophoresis. Precipitated DNA pellets from the Maxam−Gilbert sequencing reaction, with controls, were resuspended in 10 μL of gel-loading buffer [95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bromophenol blue, and 0.05% xylene cyanol FF] and 1.1 μL of 5X TBE buffer (1X TBE = 89 mM Tris-borate and 2 mM EDTA). One microliter was used to determine the amount of radioactivity (counts per minute) of each sample, and, on the basis of these values, a quantity of each sample was used for the analysis. All samples were heat denatured for 2 min at 90 °C and then loaded onto a 20% polyacrylamide denaturing gel (running buffer, 1X

TBE). Autoradiography was carried out at −80 °C using Kodak XOMAT-AR film.

RESULTS

Source of Oxidant. The xanthine oxidase/acetaldehyde (XO/AA) system is a convenient and reliable method for $O_2^{\bullet-}$/HOO$^\bullet$ generation in the absence of redox metal-catalyzed HO$^\bullet$ formation if the enzyme is dialyzed before use and metal-free buffer salts and water are employed (Britigan *et al.*, 1990; Lloyd & Mason, 1990; Aikens & Dix, 1991). The inclusion of desferal in incubations, a highly effective Fe chelator, provides an additional level of defense against HO$^\bullet$ formation. At the pH of the XO/AA experiments, approximately 0.5% of the $O_2^{\bullet-}$ is HOO$^\bullet$ (Bielski & Cabelli, 1991), and the concentration of each can be inferred from the results of a cytochrome *c* assay for $O_2^{\bullet-}$ (Experimental Procedures).

Nicking of a Radiolabeled Restriction Fragment by $O_2^{\bullet-}$/HOO$^\bullet$. To explore the mechanism of DNA cleavage by $O_2^{\bullet-}$/HOO$^\bullet$, a 5′-^{32}P-end-labeled 191 bp DNA restriction fragment was prepared. Use of this DNA substrate previously indicated that the $O_2^{\bullet-}$/HOO$^\bullet$ nicking had sequence selectivity in that only about one out of twenty sites is nicked to a large extent; further, since all four bases were sites for nicking, attack to oxidize particular bases was not the source of the selectivity. Background nicking at all bp was also observed, which is attributed to base oxidation[3] and/or attack to abstract hydrogen atoms at other sites of the deoxyribose ring by either $O_2^{\bullet-}$/HOO$^\bullet$ or secondary radicals (such as ROO$^\bullet$s) formed as intermediates on the DNA (*vide infra*). Figure 1 illustrates typical results of experiments with the radiolabeled DNA restriction fragment. Lane A is the DNA control. Lanes B and C are DNA nicking promoted by HO$^\bullet$ and ROO$^\bullet$ (produced from AAPH), respectively. Lanes D−I are experiments addressing the nicking by $O_2^{\bullet-}$/HOO$^\bullet$, as generated by the metabolism of AA by XO. Lanes D and E are the DNA incubated in the absence of XO and AA, respectively; no nicking was observed in either experiment. Lanes F−I in Figure 1 are experiments designed to examine the mechanism of nicking by $O_2^{\bullet-}$/HOO$^\bullet$. Lanes G−I are derived from incubations that contained decreasing concentrations of XO in the presence of AA (0.75, 0.5, and 0.25 unit/mL, respectively). There is no obvious dose response on the amount of cleavage as all three concentrations give roughly the same quantity of cleavage products. However, all of the AA is consumed in these experiments; thus, there are about ten HOO$^\bullet$s generated per bp, and the lack of concentration dependence serves as another control for nonspecific DNA nicking by the presence of different amounts of XO. Comparison of lanes G−I to B and C clearly demonstrates that $O_2^{\bullet-}$/HOO$^\bullet$ nicks DNA with greater and a different selectivity than do ROO$^\bullet$ and HO$^\bullet$.

Analysis of the DNA Termini. The chemical nature of the 3′-end of the 5′-end-labeled 191 bp DNA fragment after reaction with $O_2^{\bullet-}$/HOO$^\bullet$ was investigated by employing the 3′-phosphatase activity of T4 polynucleotide kinase (Cameron & Uhlenbeck, 1977). Initial 2′-, 3′-, and 4′-hydrogen atom abstractions or base oxidation followed by depurination/depyrimidination results in "blocked" 3-phosphates at the site of nicking, while 5′-hydrogen atom abstractions result in free 3′-phosphates. Initial 1′-abstractions can lead to either result, depending on the workup conditions. As a positive control, Maxam−Gilbert reactions leave the DNA with 3′-phosphate termini (Maxam & Gilbert, 1980), and after treatment with T4 polynucleotide kinase under defined conditions (Povirk & Steighner, 1990), the DNA fragment will have 3′-hydroxyl termini (Hertzberg & Dervan, 1984). 3′-Hydroxyl termini are evident in a sequencing gel by a decrease in mobility as compared to that of the 3′-phosphate termini (Hertzberg & Dervan, 1984; Povirk & Steighner,

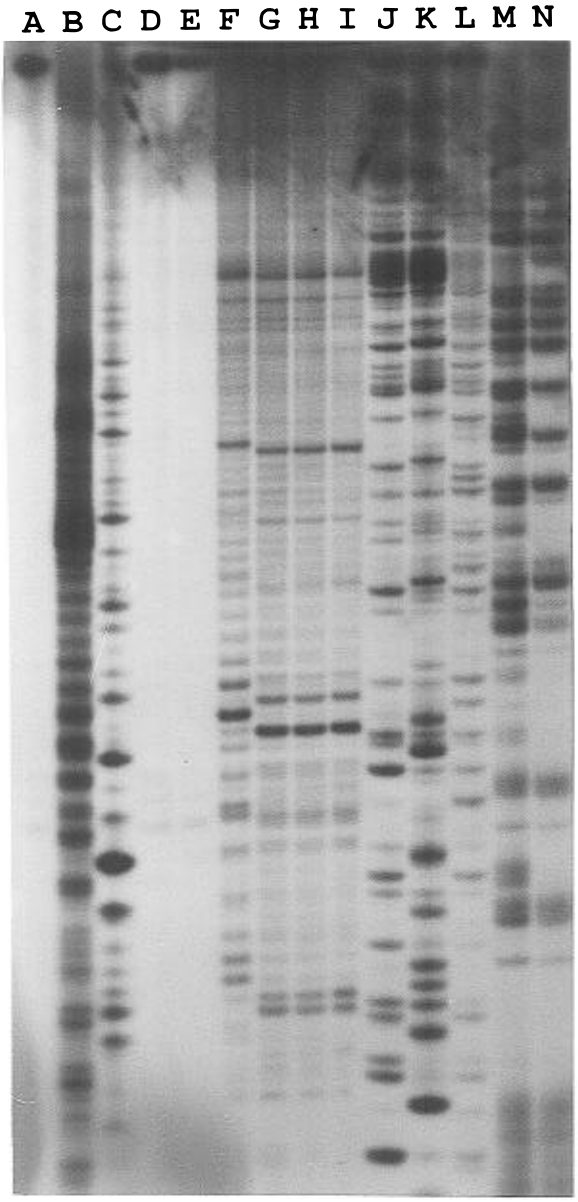

FIGURE 1: Polyacrylamide gel electrophoresis analysis of cleavage of a 5′-^{32}P-end-labeled 191 bp DNA fragment. Wells are at the top of the figure, with gel development down. For all experiments, 6.6 μM DNA ($\geq 10^6$ cpm) was incubated with the following: DNA alone (lane A), Cu/OP reagent (lane B), AAPH (1.25 μg/μL) (lane C), 20 mM AA and treated with T4 polynucleotide kinase (lane D), 0.75 unit/mL XO and treated with T4 polynucleotide kinase (lane E), 0.75 unit/mL XO/20 mM AA treated with T4 polynucleotide kinase (lane F), 0.75 unit/mL XO/20 mM AA (lane G), 0.5 unit/mL XO/20 mM AA (lane H), and 0.25 unit/mL XO/20 mM AA (lane I). The Maxam and Gilbert G sequencing reaction is in lanes J and K, the latter treated with T4 polynucleotide kinase. Lanes L−N contain the Maxam−Gilbert A+G, C+T, and C reactions, respectively.

[3] The XO/AA system as well as ROO$^\bullet$s oxidize each of the four individual deoxyribonucleoside bases to various products (T. Simandan, T. L. L. Webb, and T. A. Dix, unpublished observations).

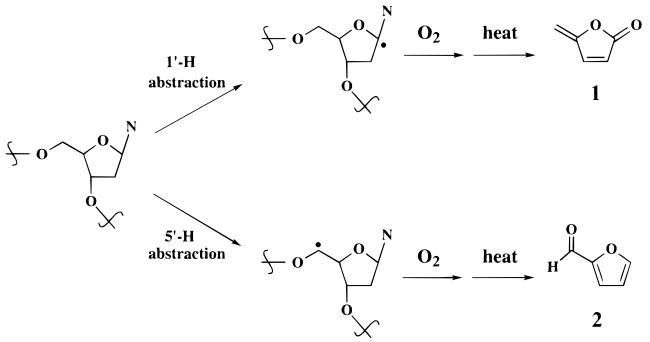

FIGURE 2: Metabolic probes for 1′- and 5′-hydrogen atom abstraction mechanisms for initiation of DNA nicking.

1990). Lane J of Figure 1 depicts the Maxam−Gilbert G reaction, while lane K depicts the same reaction with T4 polynucleotide kinase treatment. Bands in lane K are shifted up compared to the bands in lane J and indicate the presence of 3′-hydroxyl termini in lane K. Lanes L, M, and N depict the Maxam−Gilbert A+G (faint), C+T, and C reactions, respectively, to complete the sequence analysis.

Treatment of the $O_2^{\bullet-}$/HOO$^\bullet$ reaction mixtures with T4 polynucleotide kinase (lane F, Figure 1) resulted in a decreased mobility of *all* the major bands of cleavage observed in lanes G−I. This experiment indicated that all of the DNA cleavage by $O_2^{\bullet-}$/HOO$^\bullet$ results in 3′-phosphate termini and thus limits the possible mechanisms of DNA damage. This result immediately excludes 2′-, 3′-, and 4′-hydrogen atom abstractions as well as base oxidation and loss during workup as viable mechanisms for the site-selective nicking. 2′-Nicking and base oxidation/loss lead to complete blockage of the 3′-phosphates (Stubbe & Kozarich, 1987) (indeed, strand scission at apurinic/apyrimidinic sites typically must be catalyzed in the latter case by piperidine treatment during workup). Hydrogen atom abstractions at the 3′-position can result in some free 3′-phosphates, but only in the absence of solution O_2 do 3′-phosphates form exclusively (Sitlani *et al*., 1992). Abstraction of the 4′-hydrogen atom leads to both free and alkylated 3′-phosphates (Dedon *et al*., 1992); conditions that produce only free 3′-phosphates from initial 4′-attack have not been observed. Previously, it was demonstrated using this assay that attack of either HO$^\bullet$ or ROO$^\bullet$ resulted in mixed mechanisms of nicking (Dix *et al.*, 1994); both free and blocked 3′-phosphates were produced.

The formation of free 3′-phosphates at the site of DNA nicking is diagnostic of 5′-hydrogen atom abstraction for initiation of the strand scission (Goldberg, 1991). However, nicking effected by 1′-chemistry can lead to complete formation of free 3′-phosphates if incubation or workup conditions exist to catalyze release of small carbon units attached to the 3′-phosphates that are initial products of 1′-attack (Stubbe & Kozarich, 1987). To distinguish these possibilities, the formation of deoxyribose-derived products of the nicking was assayed by GC−MS. As shown in Figure 2, distinct five-carbon metabolites are formed from deoxyribose, depending on the initial site of hydrogen atom abstraction; 5-methylene-2-5*H*-furanone (**1**) is a product of 1′-hydrogen atom abstraction (Goyne & Sigman, 1987), while furfural (**2**) is a product of 5′-hydrogen atom abstraction (Pratviel *et al.*, 1991). In incubations in which $O_2^{\bullet-}$/HOO$^\bullet$ was generated, a compound was isolated that cochromatographed on GC with an authentic sample of **2**; the mass

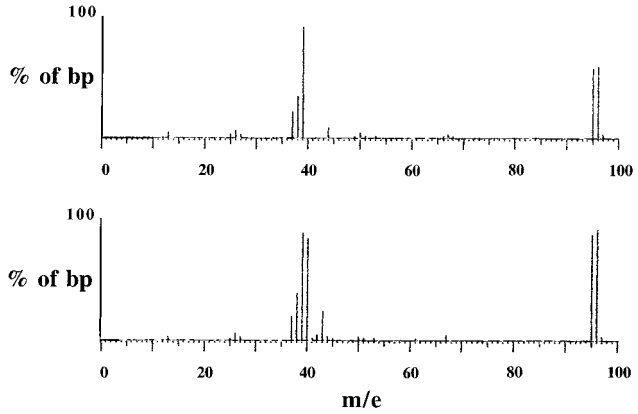

FIGURE 3: Mass spectra of an authentic sample of furfural (**2**) (top) and of the metabolite that cochromatographed with **2** (bottom).

spectrum of this compound in comparison with the mass spectrum of authentic **2** is shown in Figure 3. While the mass spectrum of **1** has the same molecular ion as that of **2**, its mass spectral breakdown pattern is very different (McLafferty & Stauffer, 1989; Pratviel *et al.*, 1991); notably, the distinct M^+, $M^+ - 1$ pattern of **2** is absent in the mass spectrum of **1**, and major ion fragmentations of **1** (*m*/*e* 68, 54, and 42) have very low populations. Metabolite **2** was not detected in experiments in which either XO or AA was omitted or if the antioxidant BHA was included in the incubation (data not shown). Both **1** and **2** could be detected by GC−MS with comparable sensitivity and at distinct retention times when spiked in control incubations. In contrast, in standard incubations, the ion fragmentations of **1** could be detected only by specific ion monitoring, while all significant ions of **2** were easily detected in the total ion profile, which indicated that **1** is at best a minor product (<1% of **2**). Thus, the identification of **2** as a product of DNA nicking by $O_2^{\bullet-}$/HOO$^\bullet$ supports the result of the 3′-phosphate assay; the initial event in the strand scission is 5′-hydrogen atom abstraction.

DISCUSSION

Mechanistic Proposal for DNA Damage by $O_2^{\bullet-}$/HOO$^\bullet$. On the basis of the above results, a mechanism for the cleavage of DNA by $O_2^{\bullet-}$/HOO$^\bullet$ can be proposed. Several mechanisms of DNA cleavage have been defined for drugs such as neocarzinostatin (Goldberg, 1991; Frank *et al.*, 1991; Sugiyama *et al.*, 1992) and bleomycin (Burger *et al.*, 1980; Giloni *et al.*, 1981; Hecht, 1986; Stubbe & Kozarich, 1987; Hamamichi *et al.*, 1992) that bind near the minor grove of DNA and degrade the deoxyribose ring. From these studies, the likely pathway for the formation of 3′-phosphate termini by $O_2^{\bullet-}$/HOO$^\bullet$-mediated damage is by initial hydrogen atom abstraction at the 5′-carbon of the deoxyribose ring (Figure 4). In the $O_2^{\bullet-}$/HOO$^\bullet$ experiments, HOO$^\bullet$ is hypothesized as the oxidant that abstracts the 5′-hydrogen atom; HOO$^\bullet$ is a fairly strong (Bielski & Cabelli, 1991) but selective (Aikens & Dix, 1991), and $O_2^{\bullet-}$ an extremely poor (Fridovich, 1986), oxidant for hydrogen atom abstraction. After reduction of the incipient 5′-deoxyribose−OO$^\bullet$ to 5′-deoxyribose−OOH, it then undergoes a spontaneous β-scission to break the DNA backbone, leaving free 3′-phosphate and forming the deoxyribose-5'-aldehyde. Heat treatment then releases furfural (**2**), which was found as a mechanistic marker in the reaction. The 5′-deoxyribose−OO$^\bullet$ may be reduced by $O_2^{\bullet-}$ or could react with the bases or deoxyribose ring to abstract hydrogen

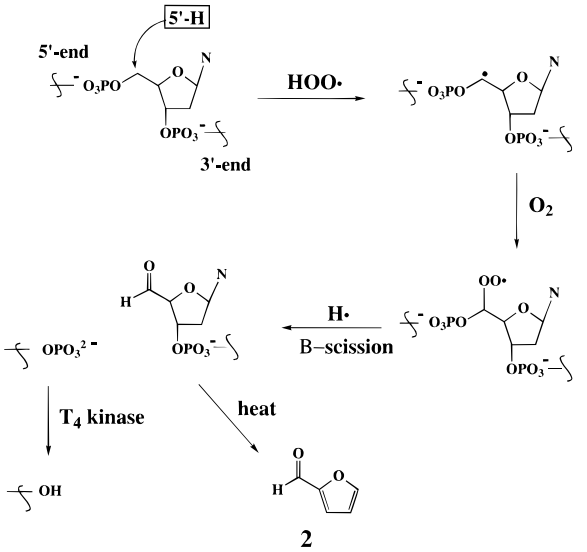

FIGURE 4: Proposed mechanism for nicking of DNA by $O_2^{\bullet-}$/HOO•, after Goldberg (1991).

atoms. The latter reactions may be a source of the nonspecific nicking observed in these experiments.

A significant body of mechanistic work on the reaction of HO• with DNA exists (von Sonntag, 1987); however, the reactivity of ROO• (in the absence of HO•) and $O_2^{\bullet-}$/HOO• is less well-understood. Results from these and previous (Dix *et al.*, 1994) experiments indicate that these key biological oxidants nick DNA by distinct mechanisms. HO• is known to nick DNA by both base oxidation and attack on deoxyribose by random hydrogen atom abstractions. ROO• nicking, while less robust, parallels HO• nicking in that multiple mechanisms are exhibited. In contrast, the $O_2^{\bullet-}$/HOO• nicking is selective for 5′-hydrogen atom abstraction, although significant background nonselective nicking also is observed. The site selectivity of DNA nicking by $O_2^{\bullet-}$/HOO•, in which, on average, one out of every twenty deoxyriboses is attacked, appears to be a result of heterogeneity in the DNA sequence that exposes only certain 5′-sites to the oxidant. ROO• is a less selective oxidant than HOO• (Aikens & Dix, 1991), while HO• is considered to be virtually nonselective (Walling, 1975). Background levels of nonspecific nicking may be attributable to base oxidations, as $O_2^{\bullet-}$/HOO• attack on each of the bases has been demonstrated;[3] hydrogen atom abstractions at other sites on the deoxyribose ring probably also contribute to the nonspecific nicking. The bases are fairly well-protected in the three-dimensional structure of intact double-stranded DNA, and DNA that is degraded more extensively may expose bases to greater attack by $O_2^{\bullet-}$/HOO•, as occurs with other oxidant-generating systems.

The previous study (Dix *et al.*, 1994) demonstrated that both $O_2^{\bullet-}$/HOO• and ROO• nick DNA to a significant degree *in vitro*, although less than comparable amounts of HO•. *In vivo*, however, kinetic- and thermodynamic-based arguments support the primary role of $O_2^{\bullet-}$ and HO• as precursors to other reduced oxygen species that are more reactive in a cellular context (Dix & Aikens, 1993). $O_2^{\bullet-}$ may exert biological damage through its conjugate acid, HOO•, while the diffusion-controlled hydrogen abstraction and addition reactions of HO• (Buxton *et al.*, 1988), each of which results in the formation of ROO•s in oxygenated solution, may indicate that the latter species are primary effectors of HO• toxicity. Since $O_2^{\bullet-}$/HOO•, ROO•, and HO• appear to damage DNA by different, characteristic, mechanisms, metabolite assays potentially could be used to define the relative activity of each oxidant *in vivo*. Furfural (**2**) has been established in this work as a product resulting from $O_2^{\bullet-}$/HOO• attack on the deoxyribose backbone of DNA. Hence, isolation of **2** from systems in which $O_2^{\bullet-}$/HOO• is generated in the vicinity of DNA would support an active role for $O_2^{\bullet-}$/HOO• in disease initiation associated with oxidant dependent DNA damage. Efforts to establish this link are in progress.

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