Peroxidase-Catalyzed Oxidative Damage of DNA and 2′-Deoxyguanosine by Model Compounds of Lipid Hydroperoxides: Involvement of Peroxyl Radicals

Waldemar Adam,* Annemarie Kurz, and Chantu R. Saha-Möller

Institut für Organische Chemie, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

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The peroxidase-catalyzed decomposition of 3-hydroperoxy-1-butene (**1**), 2,3-dimethyl-3-hydroperoxy-1-butene (**2**), *tert*-butyl hydroperoxide (**3**), ethyl oleate hydroperoxide **4**, and linoleic acid hydroperoxide **5** was applied as a chemical model system to assess whether lipid hydroperoxides may cause DNA damage under peroxidase catalysis. For this purpose, the *Coprinus* peroxidase (CIP), horseradish peroxidase (HRP), and the physiologically important lactoperoxidase (LP) were tested. Indeed, hydroperoxides **1**−**5** induce strand breaks in pBR 322 DNA upon peroxidase catalysis. For the nucleoside dG, the enzymatic decomposition of hydroperoxides **1**−**4** led to significant amounts of 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (4-HO-8-oxo-dG) and guanidine-releasing products (GRP), whereas 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG**)** was not obtained. In isolated calf thymus DNA, the efficient conversion of the guanine base (Gua) was observed. Peroxyl radicals, which are generated in situ from the hydroperoxides by one-electron oxidation with the peroxidases, are proposed as the active oxidants on the basis of the following experimental facts. (i) Radical scavengers strongly inhibit the guanine oxidation in dG and DNA and strand-break formation in the latter. (ii) EPR spectral studies with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap confirmed the formation of peroxyl radicals. (iii) The release of molecular oxygen was demonstrated, produced through the disproportionation of peroxyl radicals. The biological relevance of these findings should be seen in the potential role of the combined action of lipid hydroperoxides and peroxidases in damaging cellular DNA through peroxyl radicals.

Introduction

The peroxidation of unsaturated lipids leads to a large number of oxidation products, which include lipid hydroperoxides, as well as peroxyl and alkoxyl radicals (*1*, *2*). The lipid hydroperoxides are known to induce a range of alterations in DNA. For example, it was shown that incubation of plasmid DNA with autoxidized linoleic, linolenic, or arachidonic acid resulted in DNA strand breaks (*3*−*5*). When calf thymus DNA was exposed to lipid hydroperoxides, the guanine oxidation product 7,8-dihydro-8-oxoguanine (8-oxo-Gua)[1] was formed (*6*). Since the detected DNA alterations were enhanced by the presence of transition metals (*4*, *5*), it was concluded that these oxidations arise from the oxyl radicals generated in situ in the transition metal-catalyzed decomposition of the hydroperoxides (Fenton reaction). More recently, it was shown that the photosensitized decomposition of fatty ester hydroperoxides produces oxyl radicals, which in turn induce strand breaks in DNA (*7*).

In view of the fact that peroxidases, e.g., chloroperoxidase (*8*) and horseradish peroxidase (HRP) (*9*), release peroxyl radicals from a variety of hydroperoxides by redox chemistry, it is plausible that in cellular systems the peroxidase-catalyzed degradation of lipid hydroperoxides may generate peroxyl radicals, and these in turn cause oxidative DNA damage. To probe this possibility, we decided to investigate as a model system the peroxidase-catalyzed induction of strand breaks in supercoiled pBR 322 DNA in the presence of hydroperoxides, as well as the oxidation of the guanine base in calf thymus DNA and in the nucleoside 2′-deoxyguanosine (dG). As model compounds (Scheme 1) for the lipid hydroperoxides, we chose 3-hydroperoxy-1-butene (**1**) (*10*), ethyl oleate hydroperoxide as a 1:1 regioisomeric mixture of ethyl *trans*-9-hydroperoxyoctadec-10-enoate (**4a**) and *trans*-10-hydroperoxyoctadec-8-enoate (**4b**) (*11*), and the enantiomerically pure (*S*)-13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (**5**) (*12*). In addition, we examined *tert*-BuOOH (**3**) and 2,3-dimethyl-3-hydroperoxy-1-butene (**2**) (*13*); the latter has been previously applied as a model compound for lipid hydroperoxides (*14*). As enzymes, the *Coprinus* peroxidase (CIP) from the basidiomycete *Coprinus cinereus* (*15*, *16*), the horseradish peroxidase (HRP), and the physiologically important lactoperoxidase (LP, from bovine milk) were employed.

* To whom correspondence should be addressed. Telephone: +49-931-888-5340, ext 339. Fax: +49-931-888-4756. E-mail: adam@chemie.uni-wuerzburg.de.

[1] Abbreviations: dG, 2′-deoxyguanosine; 8-oxo-Gua, 7,8-dihydro-8-oxoguanine; HRP, horseradish peroxidase; CIP, *Coprinus* peroxidase; LP, lactoperoxidase; OC, open-circular; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; SOD, superoxide dismutase; 4-HO-8-oxo-dG, 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine; GRP, guanidine-releasing products; 8-oxo-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); oxazolone, 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentafuranosyl)-amino]-5-(2*H*)-oxazolone; imidazolone, 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one.

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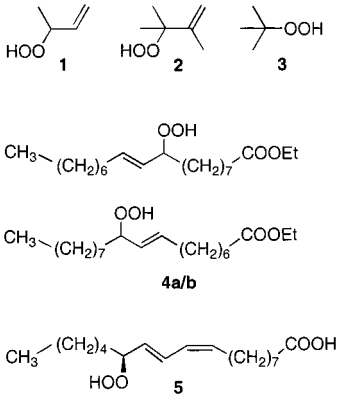
Scheme 1. Structures of Hydroperoxides 1−5



Experimental Section

Chemicals. *Coprinus* peroxidase (CIP) was obtained from Novo Nordisk as an aqueous solution (ca. 2.98 mM) with an activity of 643 kPODU/g (1 g of enzyme contains 643×10^3 peroxidase units). Horseradish peroxidase (HRP grade I, 256 units/mg of solid) was supplied by Boehringer Mannheim GmbH and superoxide dismutase (SOD from bovine erythrocytes) by Roche Diagnostics GmbH. Lactoperoxidase (LP from bovine milk, 123 units/mg of solid), bromophenol blue gel-loading solution, tris(hydroxymethyl)aminomethane (tris base), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and sodium salt of 1,2-naphthoquinone-4-sulfonic acid (NQS) were obtained from Sigma-Aldrich Chemie GmbH. Supercoiled pBR 322 DNA (form I, molecular mass of 2.9×10^6 Da, 4365 base pairs per molecule) was ordered from Pharmacia Biotech Europe GmbH. Calf thymus DNA, 2′-deoxyguanosine (dG), ethidium bromide for biochemical use, and boric acid were purchased from Merck KGaA. Agarose was acquired from Serva Feinbiochemica GmbH. 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was available from ACROS. Adenine, cytosine, guanine, thymine, ammonium formate, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were from Fluka Chemie AG. Citric acid, Na_2HPO_4, and KH_2PO_4 were from Riedel-de Haën. Methanol and acetonitrile (HPLC grade) were from Fisher Scientific GmbH. Water was deionized by employing Millipore MilliQ equipment.

Formation of Strand Breaks in Supercoiled pBR 322 DNA. The samples were prepared in a final volume of 10 μL and thermolyzed at 25−37 °C. The concentration of supercoiled pBR 322 DNA in all experiments was 10 mg/L in normal aerated 5.0 mM phosphate buffer (pH 7.4). The reactions were initiated by addition of the enzyme solution, followed by the reactant (final concentration range of 0.88−60 mM) as an acetonitrile solution (20%). Upon single-strand breakage, the supercoiled DNA is converted to the open-circular (OC) form, while double-stranded breaks lead to the linear form. After 60−160 min, to the samples was added 2.50 μL of a buffer solution with 0.05% bromophenol blue, 40% sucrose, 0.5% sodium lauryl sulfate, and 0.1 M EDTA. An aliquot (8 μL) of the resulting mixture was transferred to the 1% agarose gel, which contained 0.50 (8.0 μL) mg/L ethidium bromide. The gel electrophoresis was carried out in tris buffer (18 mM tris base, 18 mM boric acid, and 10 mM EDTA) at pH 8.0 by running the gels on a Pharmacia horizontal apparatus (GNA 100), with a power supply set at 78 V for 3 h at room temperature (ca. 20 °C). The DNA spots were detected by ethidium bromide excitation (fluorescence) with a UV transilluminator (366 nm) and recorded by photography with a Herolab camera EASY 429K, which was connected to a personal computer, equipped with a Herolab EASY software program. The ratio of open-circular and linear DNA relative to the total amount of DNA was determined from the light intensities of the spots.

HPLC Analysis. The HPLC analytical system consisted of Bischoff model 2200 analytical pumps (Bischoff GmbH, Leonberg, Germany), equipped with a Rheodyne model 7125 loop injector (Berkeley, CA) and a SpectraFlow 600 photodiode array detector (SunChrom, Friedrichsdorf, Germany), and the latter was connected in series with an ESA Coulchem model 5100A electrochemical detector, supplied with a model 5011 high-sensitivity analytical cell. For the 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (4-HO-8-oxo-dG) analysis, a Waters 994 photodiode array detector (Waters Millipore Co., Milford, MA) was used. Alternatively, a Shimadzu model RF-551 spectrofluorometric detector (Shimadzu, Kyoto, Japan) was employed, which allows the detection of guanidine-releasing products (GRP). The HPLC eluents were passed through a 0.45 μm Sartorius cellulose filter before use for degassing purposes.

The separation of 7,8-dihydro-8-oxoguanine (8-oxo-Gua) was achieved on a 250 mm × 4.6 mm (i.d.) Eurospher 100-C18 7 μm column (Knauer GmbH, Berlin, Germany) by using a mixture of 50 mM sodium citrate buffer (pH 5.0) and methanol (90:10, v/v; 1.0 mL/min) as the eluent. For the detection of 8-oxo-Gua, the oxidation potential of the electrode (E_{ox}) was set at 350 mV (*17*). The separation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) was achieved with an 80:20 mixture of sodium citrate buffer and methanol as the eluent and detected at an oxidation potential of 450 mV. The DNA bases were monitored at 254 nm by UV spectroscopy.

For the analysis of 4-HO-8-oxo-dG, a 10 mm × 4.5 mm (i.d.) Lichrospher 100-NH_2 5 μm guard column and a 250 mm × 4.5 mm (i.d.) Lichrospher 100-NH_2 5 μm column (Knauer GmbH) were used by eluting with a mixture of 25 mM ammonium formate (20%) and acetonitrile (80%) (*18*, *19*). The detection was performed at 230 nm by UV monitoring.

The guanidine-releasing products (GRP), e.g., oxazolone (for the structure, see Scheme 4), were detected by an indirect fluorescence labeling assay, after release of guanidine in alkaline solution and its condensation with 1,2-naphthoquinone-4-sulfonate (NQS). The products were separated on a 250 mm × 4.6 mm (i.d.) Eurospher 100-C18 5 μm column (Knauer GmbH) with a mixture of 25 mM ammonium formate and methanol (75:25, v/v; 1.0 mL/min) as the eluent and detected spectrofluorometrically [λ_{ex} = 355 nm, λ_{em} = 405 nm (*18*, *20*, *21*)]. The oxidation products were quantified against an external standard, and the product yields are reported as the average of at least two runs under identical conditions.

Oxidation of DNA. The reaction mixture contained 0.125 g/L calf thymus DNA (corresponds to 78.1 μM guanine) in normal aerated 5.0 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.0), 42.5 μM CIP, and 60 mM hydroperoxide in 10% acetonitrile as the cosolvent. The total volume of the reaction mixture was 400 μL. The reactions were initiated by addition of the enzyme solution, followed by the hydroperoxide solution. All experiments were performed at 37 °C, and after a selected reaction time, a 100 μL aliquot of the solution was extracted with ethyl acetate (2 × 200 μL) and lyophilized (20 °C/0.01 Torr). Treatment with 12 μL of the HF/pyridine mixture (70% HF) for 45 min at 37 °C (*22*) afforded a brown solution, which was neutralized by addition of 15 mg of calcium carbonate, suspended in 200 μL of water, and stirred vigorously for 30 min. After centrifugation (15 min at 15 000 revolutions/min) and washing of the residue with water, the combined aqueous solutions were lyophilized and dissolved in 100 μL of water prior to the quantitative determination of 8-oxo-Gua by HPLC/EC and guanine by HPLC/UV analysis.

Oxidation of 2′-Deoxyguanosine (dG). To a 0.50 mM solution of dG in 50 mM KH_2PO_4/Na_2HPO_4 buffer (pH 7.0) were added an aqueous enzyme solution and 10% of a solution of the hydroperoxide in acetonitrile. The total volume of the reaction mixture was 400 μL. The samples were thermostated at 25−37 °C. After modification of the dG, either the sample was directly analyzed by HPLC or, if necessary, a 100 μL aliquot of the solution was extracted with ethyl acetate (2 × 200 μL), lyophilized (ca. 20 °C/0.01 Torr), and dissolved in 100 μL of water prior to the quantitative determination of the dG conversion and 4-HO-8-oxo-dG yield by HPLC/UV and 8-oxo-dG by HPLC/EC analysis.

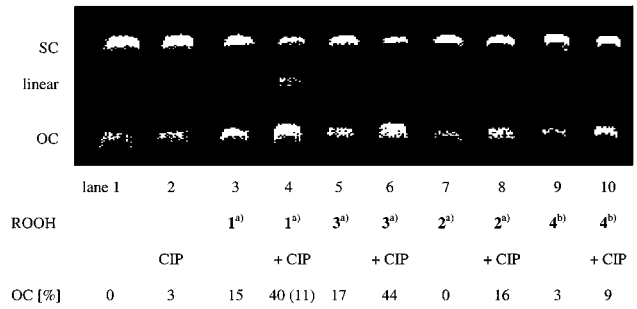


Figure 1. Gel electrophoretic analysis of strand breaks in pBR 332 DNA induced by hydroperoxides **1**−**4** under *Coprinus* peroxidase (CIP) catalysis (10 mg/L DNA in 5.0 mM KH_2PO_4 buffer at pH 7.4 with 20% CH_3CN and 0.90 μM CIP at 37 °C): (a) 60 mM ROOH **1**−**3** for 160 min and (b) 37 mM ROOH **4** for 90 min. As a blank, the DNA was thermolyzed alone (lane 1) and led to 18% open-circular (OC) DNA, which has been subtracted from the OC values (lanes 1−10) (error ± 5%); for lane 4, the linear form was also observed, given in parentheses.

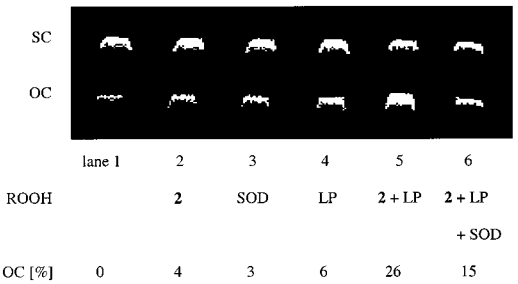


Figure 2. Gel electrophoretic analysis of strand breaks in pBR 332 DNA (10 mg/L in 5.0 mM KH_2PO_4 buffer at pH 7.4 with 20% CH_3CN at 25 °C for 4 h) induced by ROOH **2** (6.0 mM) under lactoperoxidase (LP) catalysis (0.14 μM, lane 5) and by ROOH **2**/LP in the presence of 0.64 μM superoxide dismutase (SOD, lane 6). As a blank, the DNA was thermolyzed alone (lane 1) and led to 12% open-circular (OC) DNA, which has been subtracted from the OC values (lanes 1−6) (error ± 5%).

For the analysis of the guanine moiety of dG, a 10 μL aliquot of the reaction solution was extracted with ethyl acetate (2 × 20 μL), lyophilized (ca. 20 °C/0.01 Torr), and hydrolyzed with 12 μL of the HF/pyridine mixture. The procedure described above was followed.

Assay for Guanidine-Releasing Products (GRP). For the analysis of the guanidine-releasing products (GRP), e.g., oxazolone, a 200 μL aliquot of the reaction mixture was extracted with ethyl acetate (2 × 400 μL) and dried (ca. 20 °C/0.01 Torr), dissolved in 100 μL of water, and stored for 24 h at room temperature (ca. 20 °C) to achieve complete hydrolysis of imidazolone, the oxazolone precursor (for the structures, see Scheme 4). After addition of 37 μL of aqueous NaOH (1 M) and 20 μL of an aqueous solution of NQS (0.02 M), the mixture was kept at 65 °C for 9 min in the dark. The resulting orange solution was neutralized with 43 μL of aqueous HCl (1 M) and analyzed by HPLC by employing a fluorometric detector.

EPR Studies. These studies were carried out on a Bruker EPR 420 spectrometer in a flat quartz cell (10 mm × 1 mm). Samples (volume of 800 μL) were prepared by adding, immediately before the EPR spectroscopy, a stock solution of CIP [final concentration of 57 μM in 40 mM phosphate buffer (pH 7.0)], 10 μL of the radical trap DMPO, and 80 μL of the hydroperoxide. The following spectrometer settings were employed: receiver gain, 4×10^5; modulation amplitude, 0.52 G; power, 20 mW; time constant, 163.84 ms; sweep time, 167.77 s; and sweep width, 80 G.

Results

Formation of Strand Breaks in the Peroxidase-Catalyzed Oxidation of pBR 322 DNA by Hydroperoxides 1−5. To establish whether the peroxidase-catalyzed degradation of lipid hydroperoxides may effect the oxidative damage of DNA, the DNA-cleaving properties of hydroperoxides **1**−**5** under peroxidase catalysis were first assessed. Thus, supercoiled pBR 322 DNA (10 mg/L DNA in 5.0 mM KH_2PO_4 buffer at pH 7.4, with 20% acetonitrile) was incubated with 3-hydroperoxy-1-butene (**1**, 60 mM), which served as a model compound for lipid hydroperoxides, in the presence of *Coprinus* peroxidase (CIP, 0.9 μM) at 37 °C for 160 min. The oxidation resulted in a significant amount of open-circular (OC) DNA [40%, Figure 1, lane 4; the yields of OC DNA have been corrected by subtraction of the blank (18%)] and linear DNA (11%). This is compared in Figure 1 with 3% OC DNA in the reaction with the enzyme CIP alone (Figure 1, lane 2) and with 15% OC DNA by hydroperoxide **1** alone (lane 3). Moreover, the addition of radical scavengers *i*PrOH (*23*), 2,6-di-*tert*-butyl-4-methylphenol (BHT) (*24*), and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) resulted in a significant decrease in the level of strand breaks (up to 100%, data not shown). The addition of superoxide dismutase (SOD, 0.6 μM) caused only a minor effect.

Analogous to that for 3-hydroperoxy-1-butene (**1**), the DNA-cleaving propensities for hydroperoxides **2**−**5** were assessed. Indeed, a relatively strong DNA-cleaving activity was found for tertiary hydroperoxides **2** and **3** under CIP catalysis (Figure 1, lanes 6 and 8) and a moderate activity for ethyl oleate hydroperoxide **4** (lane 10), whereas linoleic acid hydroperoxide **5** exhibited only a very weak effect (5% OC, not shown). Accordingly, the rank order of efficacy of the hydroperoxides in inducing CIP-catalyzed DNA cleavage is as follows: **1** > **3** > **2** ≫ **4** > **5**. Similar results, i.e., a substantial DNA-cleaving activity, were obtained for hydroperoxides **1**−**4** (data not shown) with HRP.

Once it was confirmed that hydroperoxides induce DNA strand breaks in the presence of *Coprinus* and horseradish peroxidases, the physiologically more important enzyme lactoperoxidase (LP) was tested. The ability of the hydroperoxide **2**/LP oxidant to induce breaks in pBR 322 DNA is displayed in Figure 2. The reaction of hydroperoxide **2** (6.0 mM) and LP (0.14 μM) with pBR 322 DNA [10 mg/L in 5.0 mM KH_2PO_4 buffer (pH 7.4) with 20% acetonitrile at 25 °C for 4 h] afforded 26% OC DNA (Figure 2, lane 5) [corrected by subtraction of the blank (lane 1), which amounted to 12% OC]. Only 4% OC DNA was obtained with hydroperoxide **2** alone (lane 2) and 6% OC DNA with LP alone (lane 4). These results clearly demonstrate the effective LP-catalyzed oxidation of DNA by the hydroperoxide **2**. In contrast, 3-hydroperoxy-1-butene (**1**) and ethyl oleate hydroperoxide **4** exhibited no DNA-cleaving ability under LP catalysis (not shown).

Oxidative Damage of dG in the Peroxidase-Catalyzed Degradation of Hydroperoxides 1−5. In addition to the DNA-cleaving activity of the hydroperoxide **1**−**5**/peroxidase systems, the oxidation of the nucleoside 2′-deoxyguanosine (dG) was investigated. Thus, 0.50 mM dG in 50 mM phosphate buffer (pH 7.0, 10% acetonitrile) was treated with 3-hydroperoxy-1-butene (**1**, 6.0 mM) in the presence of *Coprinus* peroxidase (29.8 μM) at 25 °C to establish whether the CIP-catalyzed decomposition of the hydroperoxides modifies the nucleoside by oxidation. After 170 min, the reaction

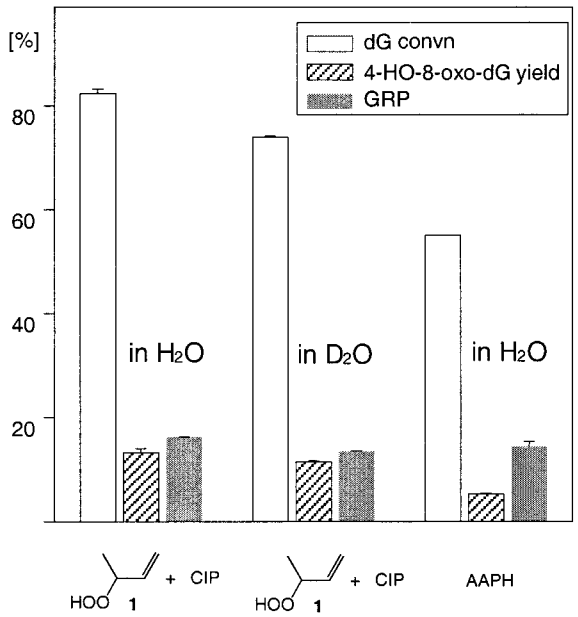


Figure 3. dG conversion and formation of 4-HO-8-oxo-dG and guanidine-releasing products (GRP) for the *Coprinus* peroxidase (CIP)-catalyzed aerobic oxidation of dG by hydroperoxide **1** in H_2O and D_2O compared with that caused by the thermal decomposition of the azoalkane AAPH in the presence of molecular oxygen. 8-Oxo-dG was not observed.

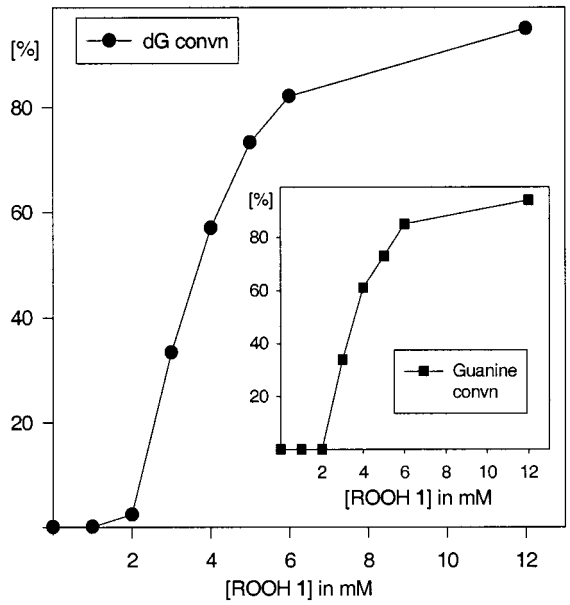


Figure 4. Concentration profile for the dG conversion by the hydroperoxide **1**/*Coprinus* peroxidase (CIP) system (0.50 mM dG in 50 mM phosphate buffer at pH 7.0 with 10% CH_3CN and 29.8 μM CIP at 25 °C for 255 min) (error ± 5%) of the stated values. The inset shows the conversion of the guanine in dG.

afforded 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxyguanosine (4-HO-8-oxo-dG) and guanidine-releasing products (GRP) in 13.3 ± 0.1% and 16.2 ± 0.8% yields at 82.4 ± 0.8% conversion of dG (Figure 3). No 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) was observed in this reaction, nor was dG consumed; only negligible amounts of the oxidation products (less than 1.2%) were detected when either CIP or hydroperoxide **1** was absent. Similar effects were obtained with the enzymes HRP and LP (data not shown), but were much less pronounced. Therefore, all subsequent dG experiments were performed exclusively with CIP.

To establish the oxidation efficacy of 3-hydroperoxy-1-butene (**1**), concentration-dependent studies were performed. The concentration profile (Figure 4) clearly shows that hydroperoxide concentrations of <2.0 mM (4 equiv) have no effect on the conversion of dG. With increasing hydroperoxide concentrations, the extent of dG conversion increases; at 6.0 mM hydroperoxide (12 equiv), more than 80% of dG was consumed. To assess whether the oxidation occurs on the guanine or on the sugar moiety of dG, the dG samples were hydrolyzed with the HF/pyridine mixture after treatment with the hydroperoxide/peroxidase oxidant and the remaining amount of guanine was determined. As the inset Figure 4 exhibits, the guanine conversion correlates exactly with the dG conversion. These results imply that the hydroperoxide **1**/CIP combination oxidizes exclusively the guanine base and not the sugar moiety of dG.

The major products in the peroxidase-catalyzed degradation of 3-hydroperoxy-1-butene (**1**) are 3-hydroxy-1-butene (**6**) and 1-buten-3-one (**7**). To assess whether these nonperoxidic compounds participate in the observed dG oxidation, both products were allowed to react with dG under the same conditions that were applied in the reaction of hydroperoxide **1**/CIP with dG. Neither of the two products caused any dG oxidation, with or without CIP.

It was important to assess whether singlet oxygen participates in the hydroperoxide **1**/CIP-mediated dG oxidation. For this purpose, the dG oxidation by the hydroperoxide **1**/CIP system was conducted in D_2O [0.50 mM dG in 50 mM phosphate buffer (pD 7.0), 10% acetonitrile, 6.0 mM **3**, and 29.8 μM CIP at 25 °C for 170 min]. Clearly, the dG oxidation is not enhanced in D_2O (Figure 3); on the contrary, in D_2O the extent of dG conversion is slightly reduced, i.e., 74.0 ± 0.2% in D_2O compared to 82.4% in H_2O. Also, the yields of 4-HO-8-oxo-dG (11.5 ± 0.2%) and guanidine-releasing products (13.5 ± 0.1%) were lower; 8-oxo-dG was not observed. These results suggest that singlet oxygen is not involved in the dG oxidation induced by the hydroperoxide **1**/CIP oxidant.

In Figure 3, the dG conversion and formation of 4-HO-8-oxo-dG and guanidine-releasing products for the CIP-catalyzed oxidation of dG in the presence of hydroperoxide **1** are compared with those caused in the thermal decomposition of the azoalkane 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH). The latter is widely used as radical initiator for the examination of in situ-generated peroxyl radical effects (*25*). The thermolysis of AAPH [2.5 mM in 50 mM phosphate buffer (pH 7.0)] in the presence of 0.50 mM dG at 40 °C for 61 h resulted in a level of dG conversion of 55.0 ± 0.5%, and 5.3 ± 0.2% 4-HO-8-oxo-dG and 14 ± 1% guanidine-releasing products were yielded; again, 8-oxo-dG was not observed. Evidently, the efficiency of dG oxidation by the hydroperoxide **1**/CIP combination is comparable to that caused by the peroxyl radical source AAPH.

To provide further evidence for the intervention of radicals as the oxidizing species in the CIP-catalyzed oxidation of dG by the hydroperoxide **1**, the inhibitory effect of the well-established radical scavengers DMPO, *tert*-BuOH (*26*), *i*PrOH (*23*), BHT (*24*), and glutathione (*27*) was examined (Figure 5). Indeed, a moderate to strong inhibitory effect was observed on the dG conversion for all of these additives, which confirms the involvement of radicals in the dG oxidation.

Table 1. Oxidation of dG by the 2,3-Dimethyl-3-hydroperoxy-1-butene (2)/*Coprinus* Peroxidase (CIP) Combination versus the H_2O_2/CIP System

entry	reagent[a]	dG conversion (%)	4-HO-8-oxo-dG (%)	GRP[b] (%)
1	CIP (59.6 μM, 48 h)	0	0.35	0.47 ± 0.02
2	ROOH **2** (30 mM, 48 h)	0	0	0.10 ± 0.01
3	ROOH **2** (30 mM)/CIP (59.6 μM, 48 h)	30 ± 3	3.9 ± 0.3	3.53 ± 0.04
4	H_2O_2 (30 mM, 84 h)	12 ± 1	nd[c]	1.22 ± 0.01
5	H_2O_2 (30 mM)/CIP (59.6 μM, 84 h)	2 ± 2	nd[c]	0.39 ± 0.07

[a] With 0.50 mM dG, 50 mM phosphate buffer (pH 7.0), and 10% CH_3CN at 37 °C. [b] Guanidine-releasing products. [c] Not determined.

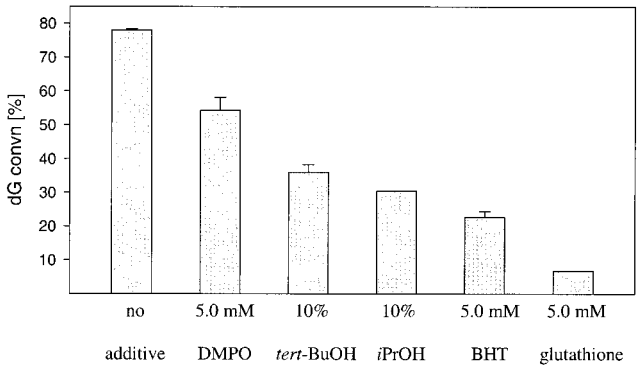


Figure 5. Effect of radical scavengers on the dG conversion for the *Coprinus* peroxidase (CIP)-catalyzed oxidation of dG in the presence of hydroperoxide **1** [0.50 mM dG in 50 mM phosphate buffer at pH 7.0 with 10% CH_3CN, 30.0 μM CIP, and 5.0 mM 3-hydroperoxy-1-butene (**1**) at 37 °C for 3 h].

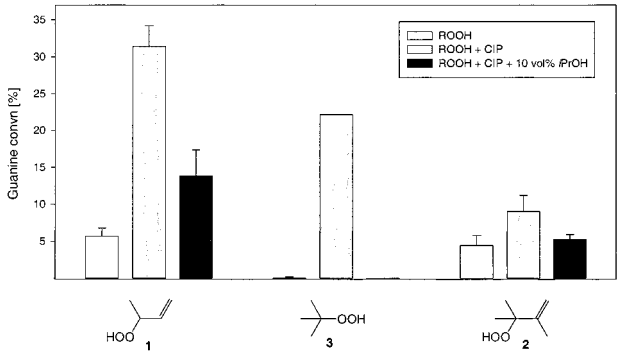


Figure 6. Guanine conversion in calf thymus DNA by ROOH (white bars), by ROOH under *Coprinus* peroxidase (CIP) catalysis (gray bars), and by the ROOH/CIP combination in the presence of 10 vol % of the radical scavenger *i*PrOH (black bars). The conditions were as follows: 0.125 g/L calf thymus DNA (corresponding to 78.1 μM guanine) in 5 mM phosphate buffer (pH 7.0), 10% CH_3CN, 42.5 μM CIP, and 60 mM ROOH at 37 °C for 23 h.

The remaining hydroperoxides **2**−**5** were also tested for their dG-oxidizing properties by CIP catalysis (data not shown). In general, tertiary hydroperoxides **2** and **3** and sterically hindered ethyl oleate hydroperoxide **4** oxidize dG in the CIP catalysis less effectively than the smaller secondary 3-hydroperoxy-1-butene (**1**). To achieve 30% dG conversion by the hydroperoxide **2**/CIP combination, a large excess of hydroperoxide (30 mM, 60 equiv) and a long reaction time (48 h) were required (Table 1, entry 3). The oxidation product 4-HO-8-oxo-dG was formed in 3.9 ± 0.3% yield and guanidine-releasing products in 3.5 ± 0.4% yield. The order of efficiency of the hydroperoxides in inducing CIP-catalyzed dG oxidation is as follows: **1** ≫ **3** > **2** > **4** ≫ **5**. Application of HPR instead of CIP produced the same trend.

The observed dG consumption by the hydroperoxide/CIP system may arise from direct oxidation of dG by the hydroperoxide-activated *Coprinus* peroxidase Compound I (CIP-I). However, no dG oxidation was observed when hydroperoxide **2** was replaced with H_2O_2 (Table 1, entry 5). Surprisingly, addition of the enzyme efficiently inhibited the low-level dG oxidation caused by H_2O_2. The activated CIP-I appears not to participate in the observed dG consumption by the hydroperoxide/CIP oxidant.

Oxidation of 8-Oxo-dG by the 3-Hydroperoxy-1-butene (1)/CIP Combination. As shown in the previous section, none of the hydroperoxide/peroxidase combinations effected the formation of dG oxidation product 8-oxo-dG. To examine whether 8-oxo-dG is further oxidized by the hydroperoxide **1**/CIP systems and, therefore, does not accumulate for detection, the authentic 8-oxo-dG [89 μM in 45 mM phosphate buffer (pH 7.0), with 11% acetonitrile] was treated at 25 °C with 5.5 mM 3-hydroperoxy-1-butene (**1**) and CIP (0.9 μM). Within 90 min, the 8-oxo-dG was completely consumed and the product 4-HO-8-oxo-dG was found in 76% yield. This result implies that 8-oxo-dG (provided that it is formed in the reaction of dG with the hydroperoxide **1**/CIP combination) may not be detected because it is quickly oxidized to 4-HO-8-oxo-dG.

Oxidative Damage of Calf Thymus DNA in the Coprinus Peroxidase (CIP)-Catalyzed Decomposition of Hydroperoxides 1−3. After the oxidation of guanine in dG by the hydroperoxide/peroxidase systems was established, the guanine oxidation in DNA was examined. Therefore, calf thymus DNA [0.125 g/L, corresponding to 78.1 μM guanine in 5.0 mM phosphate buffer (pH 7.0), with 10% acetonitrile] was incubated at 37 °C with hydroperoxides **1**−**3** (60 mM) in the presence of CIP (42.5 μM). The CIP-catalyzed degradation of hydroperoxide **1** resulted in the significant consumption of guanine [31%, Figure 6; the guanine conversion values have been corrected by subtraction of the blank values (12%)]. For *tert*-BuOOH (**3**)/CIP and hydroperoxide **2**/CIP combinations, the percent conversion values of guanine were 22 and 9, respectively. The order of efficiency of the hydroperoxides in oxidizing guanine in DNA under CIP catalysis follows the same order as in the dG case: **1** > **3** > **2**. Since the extents of DNA oxidation for the hydroperoxide **2**/CIP and **3**/CIP cases were moderate to low, experiments with hydroperoxides **4** and **5** were not pursued. Evidently, the hydroperoxide/CIP oxidant is substantially less effective in oxidizing the guanine in DNA than in dG.

Addition of the radical scavenger *i*PrOH (*23*) resulted in a significant decrease in the level of guanine conversion (Figure 6). None of the hydroperoxide/CIP combinations afforded guanine oxidation product 8-oxo-Gua. As in the case of dG, no guanine conversion in calf thymus DNA was observed when the hydroperoxides were replaced with H_2O_2 (data not shown), but again the addition of the enzyme inhibited the guanine oxidation caused by H_2O_2 alone.

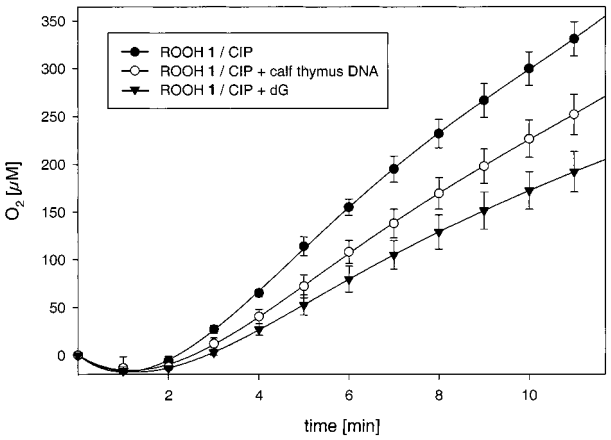


Figure 7. Evolution of oxygen gas during the 0.74 mM *Coprinus* peroxidase (CIP)-catalyzed disproportionation of 28 mM 3-hydroperoxy-1-butene (**1**, in 50 mM phosphate buffer at pH 7.0 and 20 °C) in the absence (●) and presence (○) of 0.94 mM dG and with 1.50 mg/mL (corresponding to 0.94 mM Gua) calf thymus DNA (▼).

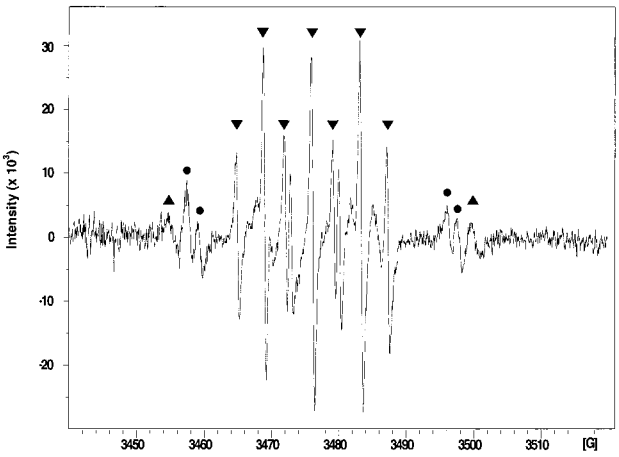


Figure 8. EPR spectrum of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO**)** radical adducts formed in the incubation of *tert*-BuOOH (**3**) with *Coprinus* peroxidase (CIP). The signals are assigned to DMPO−OOR (●) and DMPO−OH (▲) adducts (for clarity, only the outer peaks are marked) and to the DMPO oxidation product DMPOX (▼); the hyperfine coupling constants of each species and the spectrometer settings are given in the Experimental Section.

Oxygen Gas Evolution during the Coprinus Peroxidase (CIP)-Catalyzed Decomposition of 3-Hydroperoxy-1-butene (1). When CIP [0.74 mM in 50 mM phosphate buffer (pH 7.0)] was allowed to react with hydroperoxide **1** (28 mM) at room temperature, oxygen gas evolution was observed (upper curve in Figure 7), which was monitored by means of an oxygen electrode (System Clark, Bachofer GmbH, Reutlingen, Germany). In the presence of dG (0.94 mM), the hydroperoxide **1**/CIP system evolved oxygen gas less efficiently (lower curve in Figure 7). Likewise, in the presence of calf thymus DNA (1.50 mg/mL corresponds to 0.94 mM guanine), the rate of liberation of oxygen gas was reduced (middle curve in Figure 7).

EPR Studies. To provide spectral evidence for the generation of peroxyl radicals in the CIP-catalyzed decomposition of the hydroperoxides, spin trapping experiments with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in water were performed (Figure 8). The *tert*-BuOOH (**3**)/CIP combination gave a spectrum which may be assigned to a DMPO−OOR adduct, and the EPR spectral parameters are in reasonable agreement with the literature values (*9*) ($\alpha_N = 14.48$ G, $\alpha_H\beta = 10.88$ G, and $\alpha_H\gamma = 1.30$ G): $\alpha_N = 14.2$ G, $\alpha_H\beta = 10.5$ G, and $\alpha_H\gamma = 1.3$ G and $g = 2.0060$. The dominant nine-line spectrum ($\alpha_N = 7.1$ G and $\alpha_H = 4.2$ G and $g = 2.0065$) is assigned to 5,5-dimethyl-2-pyrrolidone *N*-oxyl (DMPOX) (*28*), which may arise from decomposition of the DMPO−OOR adduct (*29*) in a Kornblum−DeLaMare reaction (*30*). Indeed, the DMPO−OOR signal disappears within 15 min. The remaining signals are ascribed to a DMPO−OH or a DMPO−OR adduct **(**$\alpha_N = \alpha_H = 14.9$ G and $g = 2.0061$) (*31*). The presence of DMPO in the hydroperoxide **2**/CIP pair afforded only a weak DMPO−OOR signal (not shown). In the case of the hydroperoxide **1**/CIP combination, a complex EPR signal was recorded (not shown), which presumably derives from ring-opened decomposition products of DMPO.

Determination of the Relative Conversion Rates of Hydroperoxides 1−4 in the Peroxidase Catalysis. For this purpose, CIP [1.0 μM in 72 mM phosphate buffer (pH 7.0), with 10% acetonitrile] was added to a 2.0 mM solution of ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] and the particular hydroperoxide (1.0 mM) at 37 °C. The reaction was monitored by the change of the ABTS absorption at 405 nm for 30 min, as described in the literature (*32*). The ratio of the relative rates of CIP-catalyzed decomposition of hydroperoxides **1**, **3**, and **2** was determined to be 6:2:1. The extent of conversion of ethyl oleate hydroperoxide **4** was very low (data not shown) under these conditions, and therefore, the conversion rate could not be determined reliably. For this reason, experiments with linoleic acid hydroperoxide **5** were not pursued.

Discussion

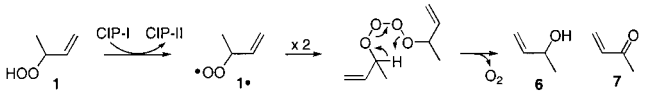
The enzymatic decomposition of hydroperoxides **1**−**5** by peroxidases such as CIP, HRP, and the mammalian lactoperoxidase (LP) induces strand breaks in pBR 322 DNA (Figures 1 and 2) and guanine conversion in both the nucleoside 2′-deoxyguanosine (dG, Figure 4) and calf thymus DNA (Figure 6). Detailed product studies have revealed that dG was oxidized by the hydroperoxide/peroxidase systems to guanidine-releasing products and 4-HO-8-oxo-dG (Figure 3), while the formation of 8-oxo-dG was not observed.

Which oxidizing species is responsible for the dG and DNA oxidation by the hydroperoxide/peroxidase oxidants? For this purpose, we will mainly focus on the 3-hydroperoxy-1-butene (**1**)/CIP system, because this combination has proven to be the most effective in the DNA cleavage and guanine oxidation.

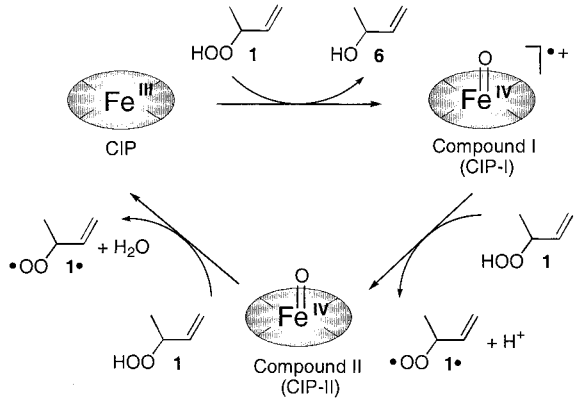
The DNA cleavage (cf. the Results) and the dG conversion (Figure 5) by the hydroperoxide **1**/CIP oxidant were efficiently inhibited by radical scavengers. These results indicate that radicals are involved in this enzymatic process. The superoxide ion may be ruled out, because the addition of SOD did not significantly reduce the level of strand break formation by the hydroperoxide **1**/CIP system (data not shown).

Are hydroxyl or alkoxyl radicals, which may be formed by the catalytic action of transition metals (Fenton-type reaction) or by homolytic cleavage of the peroxide bond, involved in the oxidation of dG and DNA? The fact that no dG oxidation was observed with the hydroperoxides in the absence of the enzyme (cf. Table 1, entries 2 and

Scheme 2. Russell Mechanism for the Formation of Molecular Oxygen from Peroxyl Radicals in the CIP-Catalyzed Oxidation of 3-Hydroperoxy-1-butene (1)



Scheme 3. Generation of Peroxyl Radicals from 3-Hydroperoxy-1-butene (1) by *Coprinus* Peroxidase (CIP)



3) argues against the generation of alkoxyl and hydroxyl radicals by homolytic cleavage of the hydroperoxides under our conditions. Moreover, no significant guanine oxidation was observed when H_2O_2 was employed instead of hydroperoxides in the presence of CIP; indeed, CIP even inhibits the low-level guanine oxidation caused by H_2O_2 itself. It has been well established that CIP possesses catalase activity (*33*), and thus, this peroxidase decomposes readily H_2O_2 and protects DNA from possible oxidative damage by H_2O_2. Therefore, it is unlikely that the peroxidase generates hydroxyl and alkoxyl radicals from the hydroperoxides (Fenton-type reaction). On the contrary, it has been reported that peroxidases release peroxyl radicals from hydroperoxides by redox chemistry (*8*, *9*, *33*).

In view of the aforementioned examples, the most likely reactive species in the peroxidase-catalyzed oxidative damage of DNA and dG by hydroperoxides are peroxyl radicals. Our EPR experiments provide evidence for this, in accord with literature reports (*8*, *9*). As shown in the EPR spectrum (Figure 8) for the spin trapping with DMPO, a weak signal for the peroxyl radical DMPO−OOR adduct is observed for the *tert*-BuOOH (**3**)/CIP system; however, the major signal is due to the DMPOX species (*9*). The latter is the decomposition product of the DMPO−OOR adduct (*29*), formed through base-catalyzed elimination of ROH in a Kornblum−DeLaMare reaction (*30*). Indeed, the DMPO−OOR adduct is a transient species, and the weak EPR signal is no longer observed after 15 min.

In the reaction of CIP with hydroperoxide **1**, the evolution of oxygen gas is observed (Figure 7), which arises from the disproportionation of peroxyl radicals according to the Russell mechanism (*34*), as shown in Scheme 2. The generation of the peroxyl radicals **1**• (Scheme 3) is rationalized in terms of one-electron oxidation of hydroperoxide **1** by the hydroperoxide-activated enzyme (Compound I and II). In the presence of dG or calf thymus DNA, the evolution of oxygen gas from the hydroperoxide **1**/CIP system was significantly inhibited (Figure 7). The lower rate of O_2 release in the presence of dG or DNA may be due to the reaction of peroxyl radicals with guanine in generating the guanine radical (addition, H abstraction). The resulting guanine radicals trap the molecular oxygen generated in the disproportionation of peroxyl radicals. Additionally, due to their reaction with guanine, fewer peroxyl radicals are available for dimerization and release of O_2 through the Russell mechanism (Scheme 2).

The inhibition of oxygen evolution by dG is more effective than the inhibition by calf thymus DNA. This experimental fact is consistent with the results depicted in Figures 4 and 7, in that the oxidation of dG by the hydroperoxides under CIP catalysis is much more pronounced than the oxidation of calf thymus DNA. Unfortunately, to date no rate constants of the reaction of peroxyl radicals with DNA or dG are known. For comparison, reactive oxygen species such as singlet oxygen react in water with dG (*35*) approximately 10 times faster than with DNA (*36*, *37*); the same is valid for the hydroxyl radical (*38*). We assume that the reaction of peroxyl radical **1**• with dG is likewise faster than with the calf thymus DNA. In the case of DNA, its oxidation by peroxyl radical **1**• is supposedly slow, and other reaction pathways, namely, the disproportionation of the peroxyl radicals, may compete with the DNA reaction.

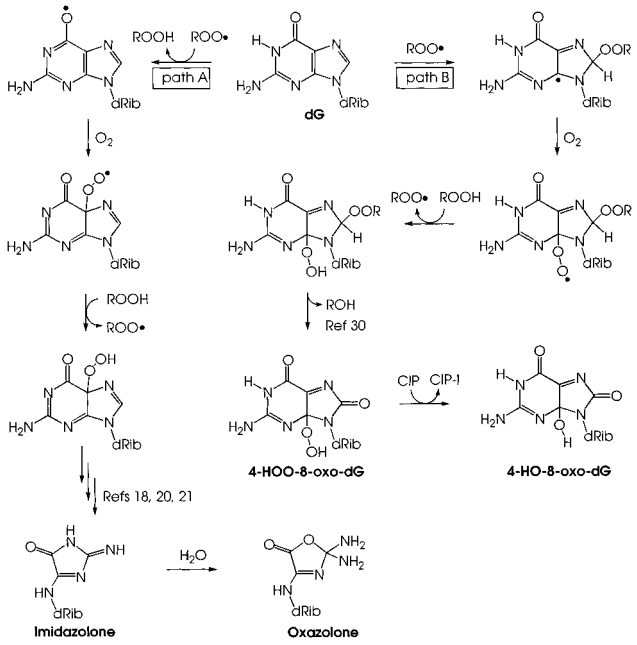
As for the possible participation of singlet oxygen as an oxidant, previously it was shown that about 12% of the released molecular oxygen in the peroxidase-catalyzed decomposition of hydroperoxides is electronically excited singlet oxygen (*39*). The lifetime of singlet oxygen is ca. 10-fold higher in D_2O than in H_2O (*40*, *41*); therefore, if singlet oxygen were involved in the present case, the reaction in D_2O would enhance DNA and dG oxidation. As shown in Figure 3, the dG conversion and formation of the guanidine-releasing products, as well as the acclaimed characteristic $^1O_2$ product 4-HO-8-oxo-dG (*42*), are not enhanced in D_2O versus H_2O. Clearly, in the present case, singlet oxygen does not play a significant role in the observed DNA and dG oxidation.

To provide additional evidence that peroxyl radicals are involved in the peroxidase-catalyzed DNA and dG oxidation by hydroperoxides, studies with the azoalkane AAPH have been performed. The latter was recently used for probing peroxyl radical effects in DNA and dG damage (*43*−*46*). As shown in Figure 3, the dG oxidation to guanidine-releasing products and 4-HO-8-oxo-dG in the thermolysis of AAPH is comparable to that caused by the hydroperoxide **1** under CIP catalysis. These AAPH results confirm that peroxyl radicals are responsible for the peroxidase-mediated oxidative damage of dG and DNA by hydroperoxides observed herein.

The order of efficiency of the hydroperoxides in inducing CIP-catalyzed dG oxidation by peroxyl radicals is as follows: **1** > **3** > **2** > **4** ≫ **5** (cf. the Results). In general, the acceptance of sterically hindered and, particularly, tertiary hydroperoxides by HRP (*47*, *48*) and CIP (*33*) is poor; thus, this reactivity order of hydroperoxides **1**−**4** reflects presumably the ease of substrate acceptance by the enzymes. Indeed, a control experiment (cf. the Results) corroborates that the reactivity order is consistent with the relative rates of the CIP-catalyzed decomposition of hydroperoxides **1**−**4**.

For the most effective peroxyl radical source, namely, the hydroperoxide **1**/CIP system, the yield of OC DNA (single strand breaks) was 40%. For a neutral peroxyl

Scheme 4. Proposed Mechanism for the Oxidation of dG to the Guanidine-Releasing Product Oxazolone (path A) and to the 4-HO-8-oxo-dG (path B) by Peroxyl Radicals



radical, as in this case, this presents a substantial DNA cleaving activity (*46*). Since hydroperoxide **1** in combination with CIP oxidized only the guanine base, and not the sugar moiety of the isolated nucleoside dG (Figure 4), it is not likely that the observed DNA strand scission arises from the direct attack of the peroxyl radicals on the deoxyribose. We propose that the strand breaks are formed by oxidative modifications of the nucleobases (*49*).

Now the following question arises: how are products 4-HO-8-oxo-dG and GRP (e.g., oxazolone) formed in the oxidation of dG by peroxyl radicals? A plausible mechanism is proposed in Scheme 4, based on literature speculation and the experimental results presented here. The oxidation of dG to the guanidine-releasing product oxazolone may proceed through hydrogen abstraction (Scheme 4, path A) by peroxyl radicals to the guanosine radical. The latter may trap molecular oxygen and form oxazolone in several steps, as described elsewhere (*18*, *20*, *21*). The oxidation of dG to 4-HO-8-oxo-dG may be initiated by the addition of the peroxyl radical (Scheme 4, path B) at the C8 position of dG (*50*). The resulting dG radical species may trap molecular oxygen to yield a peroxyl radical, which in turn abstracts a hydrogen atom from the employed hydroperoxide. The resulting dG hydroperoxide eliminates alcohol under base catalysis in a Kornblum−DeLaMare reaction (*30*) to yield 4,8-dihydro-4-hydroperoxy-8-oxo-2′-deoxyguanosine (4-HOO-8-oxo-dG). In the final step, the 4-HOO-8-oxo-dG is reduced by CIP to 4-HO-8-oxo-dG under generation of the activated CIP I complex.

Conclusions

From our results, we conclude that peroxidases release peroxyl radicals from hydroperoxides, which in turn oxidize the guanine in both dG and DNA, and induce strand breaks in the latter. The following experimental facts support this conclusion. (i) EPR spectral evidence was provided for the formation of peroxyl radicals in the catalytic decomposition of hydroperoxides **2** and **3** by CIP. (ii) In the reaction of CIP with hydroperoxide **1**, the evolution of oxygen gas was observed; the O_2 must come from the hydroperoxide, since it has been established that the CIP/ROOH mixture releases O_2 through the disproportionation of peroxyl radicals according to the Russell mechanism. (iii) The dG oxidation in the thermolysis of the azoalkane AAPH is comparable to that caused by the hydroperoxide **1**/CIP combination. (iv) Control experiments have revealed that other oxidizing species, such as singlet oxygen, superoxide ion, and hydroxyl and alkoxyl radicals, and the activated CIP Compound I are not involved.

The results of this chemical study might have biological relevance, since 3-hydroperoxy-1-butene (**1**), which in combination with CIP proved to be the most effective oxidant, contains an allylic hydroperoxy group analogous to that in naturally occurring lipid hydroperoxides.

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