J|A|C|S
ARTICLES
Published on Web 03/07/2002

Horseradish Peroxidase Catalyzed Nitric Oxide Formation from Hydroxyurea

Jinming Huang,[‡] Erin M. Sommers,[‡] Daniel B. Kim-Shapiro,[†] and S. Bruce King*[,‡]

Contribution from the Departments of Chemistry and Physics, Wake Forest University, Winston-Salem, North Carolina 27109

Received October 1, 2001. Revised Manuscript Received January 9, 2002

Abstract: Hydroxyurea represents an approved treatment for sickle cell anemia and a number of cancers. Chemiluminescence and electron paramagnetic resonance spectroscopic studies show horseradish peroxidase catalyzes the formation of nitric oxide from hydroxyurea in the presence of hydrogen peroxide. Gas chromatographic headspace analysis and infrared spectroscopy also reveal the production of nitrous oxide in this reaction, which provides evidence for nitroxyl, the one-electron reduced form of nitric oxide. These reactions also generate carbon dioxide, ammonia, nitrite, and nitrate. None of these products form within 1 h in the absence of hydrogen peroxide or horseradish peroxidase. Electron paramagnetic resonance spectroscopy and trapping studies show the intermediacy of a nitroxide radical and a C-nitroso species during this reaction. Absorption spectroscopy indicates that both compounds I and II of horseradish peroxidase act as one-electron oxidants of hydroxyurea. Nitroxyl, generated from Angeli's salt, reacts with ferric horseradish peroxidase to produce a ferrous horseradish peroxidase−nitric oxide complex. Electron paramagnetic resonance experiments with a nitric oxide specific trap reveal that horseradish peroxidase is capable of oxidizing nitroxyl to nitric oxide. A mechanistic model that includes the observed nitroxide radical and C-nitroso compound intermediates has been forwarded to explain the observed product distribution. These studies suggest that direct nitric oxide producing reactions of hydroxyurea and peroxidases may contribute to the overall pharmacological properties of this drug.

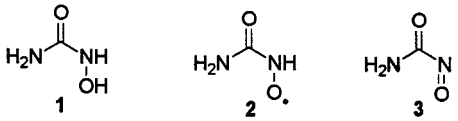
Hydroxyurea (**1**) blocks DNA synthesis by inhibiting ribonucleotide reductase and represents an effective treatment for a number of cancers, especially leukemias.[1] Such activity has led to the suggestion of hydroxyurea as a possible HIV therapy.[2] Hydroxyurea has also recently emerged as a new approved treatment for sickle cell disease.[3,4] The beneficial effects of hydroxyurea treatment to the sickle cell patient appear to result from an increase in the production of fetal hemoglobin, a genetically distinct hemoglobin that prevents the polymerization of deoxy sickle cell hemoglobin.[3,4] However, some sickle cell patients benefit from hydroxyurea treatment before their levels of fetal hemoglobin increase, indicating that the positive effects of hydroxyurea cannot be completely explained by an increase in fetal hemoglobin.[3−6] Despite the use of hydroxyurea as a treatment for these various conditions, clear molecular mechanisms describing hydroxyurea's actions remain to be described.

Recent reports indicate that hydroxyurea acts as a source of nitric oxide (NO) under oxidative conditions. Nitric oxide plays a direct role in the maintenance of normal blood pressure and flow, the immune response, and neurotransmission.[7] Treatment of hydroxyurea with hydrogen peroxide and copper(II) sulfate produces a "NO-like" species capable of nitrosating morpholine that eventually decomposes to nitrite (NO_2^-) and nitrate (NO_3^-), the stable oxidative decomposition products of NO.[8] Electron paramagnetic resonance (EPR) studies demonstrate that the chemical oxidation of hydroxyurea with hydrogen peroxide or copper(II) sulfate in aqueous dimethyl sulfoxide produces the nitroxide radical (**2**).[9] Treatment of hydroxyurea with hydrogen peroxide and heme proteins produces the nitroxide radical (**2**), NO_2^-, and NO as judged by EPR experiments with use of an NO-specific trap.[10] Similar EPR studies show the production of NO during the oxidation of hydroxyurea with hydrogen peroxide in the presence of various copper-containing enzymes.[11] The reaction of hydroxyurea and bovine oxyhemoglobin (oxyHb, Fe^{2+}-O_2) forms **2** and iron nitrosyl hemoglobin (HbNO, Fe^{2+}-NO).[12] Recent EPR studies demonstrate the in

* To whom correspondence should be addressed. E-mail: kingsb@wfu.edu.
‡ Department of Chemistry, Wake Forest University.
† Department of Physics, Wake Forest University.

(1) Yarbro, J. W. *Semin. Oncol.* **1992**, *19*, 1−10.
(2) Lori, F.; Malykh, A.; Cara, A.; Sun, D.; Weinstein, J. N.; Lisziewicz, J.; Gallo, R. C. *Science* **1994**, *266*, 801−805.
(3) Charache, S.; Terrin, M. L.; Moore, R. D.; Dover: G. J.; Barton, F. B.; Eckert, S. V.; McMahon, R. P.; Bonds, D. R. (and the Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia) *N. Engl. J. Med.* **1995**, *332*, 1317−1322.
(4) Charache, S.; Barton, F. B.; Moore, R. D.; Terrin, M. L.; Steinberg, M. H.; Dover, G. J.; Ballas, S. K.; McMahon, R. P.; Castro, O.; Oswaldo, E. P. (and the Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia) *Med.* **1996**, *75*, 300−326.
(5) Schechter, A. N.; Rodgers, G. P. *New Engl. J. Med*. **1996**, *334*, 333−334.
(6) Charache, S. *Semin. Hematol.* **1997**, *34, No. 3., Suppl. 3*, 15−21.
(7) Kerwin, J. F., Jr.; Lancaster, J. R., Jr.; Feldman, P. L. *J. Med. Chem*. **1995**, *38*, 4343−4462.
(8) Kwon, N. S.; Stuehr, D. J.; Nathan, C. F. *J. Exp. Med*. **1991**, *174*, 761−767.
(9) Lassmann, G.; Liermann, B. *Free Radical Biol. Med.* **1989**, *6*, 241−244.
(10) Pacelli, R.; Taira, J.; Cook, J. A.; Wink, D. A.; Krishna, M. C. *The Lancet* **1996**, *347*, 900.
(11) Sato, K.; Akaike, T.; Sawa, T.; Miyamoto, Y.; Suga, M.; Ando, M.; Maeda, H. *Jpn. J. Cancer Res*. **1997**, *88*, 1199−1204.
(12) Stolze, K.; Nohl, H. *Biochem. Pharmacol*. **1990**, *40*, 799−802.

Chart 1



vivo formation of HbNO in the blood of both rats and humans upon administration of hydroxyurea.[13,14] Under physiological conditions, HbNO reportedly exists in an oxygen-dependent equilibrium with the vasorelaxant protein *S*-nitrosohemoglobin (SNO-Hb, *S*-nitrosated on the β-93 cysteine residues).[15,16] As NO also inhibits ribonucleotide reductase and both NO and HbNO have been proposed as potential therapies for sickle cell disease,[17−20] the in vivo formation of NO from hydroxyurea could explain a portion of hydroxyurea's effects and define new NO-based strategies for treatment of these conditions.We previously demonstrated hydrogen peroxide slowly oxidizes hydroxyurea to produce nitrous oxide (N_2O), evidence for nitroxyl or the nitroxyl ion (HNO/NO^-).[21] Adventitious metal ions most likely assist this process in which trapping experiments reveal the C-nitroso species (**3**) as an intermediate.[21] In addition, ferric heme groups oxidize hydroxyurea to produce an iron−NO complex and ferric heme-containing proteins, such as methemoglobin (metHb, Fe^{3+}), react with hydroxyurea in vitro to form HbNO.[22,23] Such results prompted our investigation of whether peroxidases, iron heme containing enzymes activated by hydrogen peroxide, could act as catalysts for NO release from hydroxyurea. Here we demonstrate the ability of horseradish peroxidase (HRP) to catalyze both NO and HNO production from hydroxyurea. Furthermore, EPR spectroscopy and trapping studies reveal the presence of both the nitroxide radical (**2**) and the C-nitroso compound (**3**) during this reaction. Absorption spectroscopy shows hydroxyurea reduces both compounds I and II, the reactive oxidative intermediates of the HRP reaction cycle. Both absorption and EPR spectroscopy identify ferric HRP and possibly compounds I and II as oxidants capable of converting HNO to NO and producing a ferrous HRP−NO complex. Together these results allow the development of a mechanistic model to explain product formation during these reactions.

Materials and Methods

Materials. Hydroxyurea, 30% hydrogen peroxide, potassium cyanide, and horseradish peroxidase (Type I) were purchased from Sigma Chemical Company, St. Louis, MO. 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5,-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide, potassium salt (Carboxy-PTIO) and sodium trioxodinitrate (Angeli's salt) were purchased from Cayman Chemical Company, Ann Arbor, MI. 1,3-Cyclohexadiene was purchased from Aldrich Chemical Co., Milwaukee, WI.

Horseradish Peroxidase Catalyzed Oxidation of Hydroxyurea. Hydrogen peroxide (30%, 12.5 μL, 0.1 mmol, 50 mM) was added to a solution of hydroxyurea (7.6 mg, 0.1 mmol, 50 mM) and HRP (3.1 mg, 11.5 μM) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature. The concentration of HRP was determined by measuring the absorbance at 402 nm in a 0.5 cm path-length cell and using an extinction coefficient of $\epsilon = 9.5 \times 10^4\ M^{-1}\ cm^{-1}$.[24] UV−vis measurements were taken on either a Hewlett-Packard 8452a diode array spectrophotometer (Wilmington, DE) or a Cary 100 Bio UV−visible spectrophotometer (Varian, Walnut Creek, CA). The flask was capped with a rubber septum and reaction products were analyzed after 1 and 24 h. Each reaction was performed in triplicate and the yields averaged. An identical reaction was run in the presence of KCN (6.5 mg, 0.1 mmol), and the products were analyzed in a similar fashion.

Gas Chromatographic Analysis of Reaction Headspace. After 1 and 24 h, an aliquot of the reaction headspace (250 μL) was injected onto a 6890 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, a 6 ft × 1/8 in. Porapak Q column at an operating oven temperature of 50 °C (injector and detector 150 °C) with a flow rate of 16.67 mL/min (He, carrier gas). The retention times of carbon dioxide and nitrous oxide were 2.14 and 2.78 min, respectively, and identical to known samples of CO_2 and N_2O (Aldrich). The yields were calculated on the basis of a standard curve prepared by injecting known amounts of these gases.

Gas Infrared Spectroscopy of Reaction Headspace. After 24 h, the reaction headspace was transferred by a gastight syringe to an argon-flushed flow-through gas infrared (IR) spectroscopy cell (10 cm) equipped with NaCl plates as windows sealed with beeswax. An IR spectrum was recorded for each reaction on a Mattson 4020 Galaxy Series FT-IR spectrophotometer. Standard spectra were obtained from known samples of these gases (Aldrich). ^{15}N-Hydroxyurea used in these reactions was prepared as previously described.[25]

Nitrite and Nitrate Analysis. An aliquot (5 μL) of the reaction mixture was injected into the reaction vessel of a Sievers 280 nitric oxide analyzer chemiluminescence detector. This apparatus directly detects NO and can be used for nitrite and nitrate analysis under conditions where these species are converted to NO. For nitrite analysis, the reaction vessel contained 1% w/v KI in glacial acetic acid to reduce nitrite to NO. For nitrate analysis, the reaction vessel contained a solution of vanadium(III) chloride in hydrochloric acid at 90 °C that reduces both nitrite and nitrate to nitric oxide. The nitrate concentration is thus obtained by subtracting the measured nitrite concentration from the concentration of nitrite and nitrate. The concentrations of nitrite and nitrate were determined based on a standard curves.

Ammonia Analysis. Ammonia was detected and quantified with use of a commercial ammonia test kit (Sigma 171-A). This method for ammonia detection is based on the glutamate dehydrogenase catalyzed reductive amination of 2-oxogluatarate by NADPH. The decrease in absorbance at 340 nm due to the oxidation of NADPH is proportional to the ammonia concentration.

Trapping of Cycloadduct (4). 1,3-Cyclohexadiene (19.0 μL, 0.1 mmol, 50 mM) was added to a solution of hydroxyurea (7.6 mg, 0.1 mmol, 50 mM), hydrogen peroxide (30%, 12.5 μL, 0.1 mmol, 50 mM), and HRP (3.1 mg, 11.5 μM) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature. The reaction was monitored by TLC and after 1 h the reaction mixture was extracted with EtOAc (2 × 2.0 mL), dried over $MgSO_4$, and evaporated to give cycloadduct (**4**) as a residue: TLC R_f 0.26; ^1H NMR (300 MHz, $CDCl_3$, TMS internal standard) δ 6.4 (m, 2H), 4.7 (s, 1H), 4.9 (s, 1H), 1.4 (m, 4H).[26]

(13) Jiang, J.; Jordan, S. J.; Barr, D. P.; Gunther, M. R.; Maeda, H.; Mason, R. P. *Mol. Pharmacol.* **1997**, *52*, 1081−1086.
(14) Glover, R. E.; Ivy, E. D.; Orringer, E. P.; Maeda, H.; Mason, R. P. *Mol. Pharmacol.* **1999**, *55*, 1006−1010.
(15) Gow, A. J.; Stamler, J. S. *Nature* **1998**, *391*, 169−173.
(16) Stamler, J. S.; Jia, L.; Eu, J. P.; McMahon, T. J.; Demchenko, I. T.; Bonaventura, J.; Gernert, K.; Piantadosi, C. A. *Science* **1997**, *276*, 2034−2037.
(17) Lepoivre, M.; Flaman, J. M.; Bobe, P.; Lemaire, G.; Henry, Y. *J. Biol. Chem.* **1994**, *269*, 21891.
(18) Head, C. A.; Brugnara, C.; Martinez-Ruiz, R.; Kacmarek, R. M.; Bridges, K. R.; Kuter, D.; Bloch, K. D.; Zapol, W. M. *J. Clin. Invest.* **1997**, *100*, 2293−1198.
(19) McDade, W. A.; Shaba, H. M.; Carter, N. *Biophys. J.* **1996**, *72*, A9.
(20) Nagel, R. L. *J. Clin. Invest.* **1999**, *104*, 847−848.
(21) Xu, Y.; Mull, C. D.; Bonifant, C. L.; Yasaki, G.; Palmer, E. C.; Shields, H.; Ballas, S. K.; Kim-Shapiro, D. B.; King, S. B. *J. Org. Chem.* **1998**, *63*, 6452−6453.
(22) Kim-Shapiro, D. B.; King, S. B.; Shields, H.; Kolibash, C. P.; Gravatt, L.; Ballas, S. K. *Biochim. Biophys. Acta* **1999**, *1428*, 381−387.
(23) Rupon, J. W.; Domingo, S. R.; Smith, S. V.; Gummadi, B. K.; Shields, H.; Ballas, S. K.; King, S. B.; Kim-Shapiro, D. B.*Biophys. Chem.* **2000**, *84*, 1−11.
(24) Mauk, M. R.; Girotti, A. W. *Biochemistry* **1974**, *13*, 1757−1763.
(25) Yasaki, G.; Xu, Y.; King, S. B. *Synth. Commun.* **2000**, *30*, 2041−2047.
(26) Fleming, S. A.; Keck, G. A. *Tetrahedron Lett.* **1978**, *48*, 4763−4766.

EPR Detection of Nitroxide Radical (3). A solution of hydroxyurea (500 mM), hydrogen peroxide (290 mM), and HRP (30 μM) in phosphate buffer (100 mM, pH 7.4) was placed in a flat EPR tube. EPR spectra were taken on a Bruker ER200D spectrometer using 13.4 mW microwave power, 1.25 G modulation amplitude, and a 9.80 GHz microwave frequency at room temperature.

Chemiluminescence NO Detection. A solution of hydroxyurea (1.0 mM) and horseradish peroxidase (0.2 μM) was placed directly into the sealed reaction vessel of a Sievers 280 nitric oxide analyzer chemiluminescence detector that was continuously purged with argon. To this solution, aliquots (5.0 and 10.0 μL) of H_2O_2 (5.0 mM) were injected and the current was recorded.

EPR Detection of NO with 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5,-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide, Potassium Salt (Carboxy-PTIO). A solution of hydroxyurea (50 mM), HRP (11.5 μM), and Carboxy-PTIO (0.20 μM) in phosphate buffer (100 mM, pH 7.4, 0.3 mL) was placed in a flat EPR tube. A control spectrum was taken on a Bruker ER200D spectrometer at room temperature using 8.5 mW microwave power, 1.25 G modulation amplitude, and 9.8 GHz microwave frequency. A solution of hydrogen peroxide (50 mM) in phosphate buffer (100 mM, pH 7.4) was then added to this mixture and spectra were recorded on the same instrument under similar conditions over the next 40 min. Similarly, a solution of sodium trioxodinitrate (3 μL, 0.0003 mmol, 1.0 mM) in 0.01 M NaOH was added to a solution of HRP (3.1 mg, 11.5 μM) and Carboxy-PTIO (4.0 μM) in the absence or presence of hydrogen peroxide (1.0 mM) in phosphate buffer (100 mM, pH 7.4, 0.3 mL) at room temperature in a flat EPR tube. EPR spectra of these solutions were taken on a Bruker ER200D spectrometer at room temperature using 8.5 mW microwave power, 1.25 G modulation amplitude, and 9.8 GHz microwave frequency.

UV−Vis and EPR Detection of Ferrous HRP−NO from the Reaction of Sodium Trioxodinitrate with Horseradish Peroxidase. A solution of sodium trioxodinitrate (20 μL, 0.002 mmol, 1.0 mM) in 0.01 M NaOH was added to a degassed solution of HRP (3.1 mg, 11.5 μM) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature in a cuvette. UV−vis measurements were taken every minute on a Cary 100 Bio UV−visible spectrophotometer (Varian, Walnut Creek, CA). Similarly, a solution of sodium trioxodinitrate (15 μL, 0.0015 mmol, 5.0 mM) in 0.01 M NaOH was added to a degassed solution of HRP (20.9 mg, 500 μM) in phosphate buffer (100 mM, pH 7.4, 0.3 mL) at room temperature in an EPR tube and frozen in liquid nitrogen (77 K). EPR spectra were taken on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency. The *g*-values were determined from a superimposed spectrum of 2,2-diphenyl-1-picrylhydrazyl, $g = 2.0036$.

Results

Addition of hydrogen peroxide (50 mM) to a pale yellow solution of hydroxyurea (50 mM) and horseradish peroxidase (HRP, 11.5 μM) in phosphate buffer produces a reddish colored solution within 1 min. Over the next 10 min, the color of the solution returns to a pale yellow, similar to that of the starting solution, with the noticeable evolution of gas. Absorption measurements show the characteristic Soret absorbance for ferric horseradish peroxidase at 402 nm in the original solution shifts to 419 nm upon hydrogen peroxide addition and then returns to 402 nm over time (Figure 1).[27] The absorption spectrum of the final reaction mixture appears similar to the initial spectrum of ferric HRP. Addition of hydrogen peroxide (50 mM) to a solution of HRP (11.5 μM) in the absence of hydroxyurea also produces a reddish colored solution characterized by an absorbance at 419 nm within 1 min that remains stable over 10 min. Gas evolution also greatly diminishes in the absence of hydroxyurea. Addition of hydroxyurea (50 mM) to this solution shifts the absorbance back to 402 nm. No changes occur in the HRP absorption spectrum upon addition of hydroxyurea to a solution of HRP in the absence of hydrogen peroxide (Figure 1).

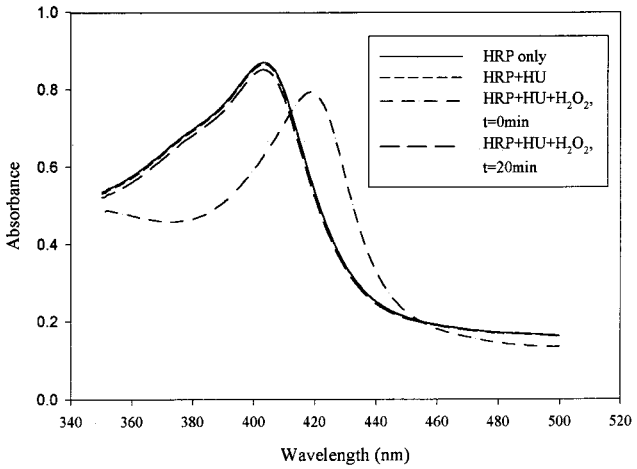


Figure 1. Absorbance spectra of ferric HRP (solid line), HRP and hydroxyurea (short dashed line), HRP and hydroxyurea immediately after hydrogen peroxide addition (dash−dot line), and HRP, hydroxyurea, and hydrogen peroxide after 20 min (long dashed line).

Gas chromatographic analysis of the headspace above a solution of hydroxyurea, hydrogen peroxide, and HRP after 1 h reveals the presence of nitrous oxide (N_2O, 20%) and carbon dioxide (CO_2, 21%). Gas-phase infrared spectroscopy of the reaction headspace confirms the formation of these gases by showing characteristic peaks at 2363 cm^{-1} for CO_2 and 2223 and 1284 cm^{-1} for N_2O.[28] Substituting ^{15}N-hydroxyurea for ^{14}N-hydroxyurea in this reaction shifts the peaks for N_2O to 2154 and 1265 cm^{-1}, indicative of $^{15}N^{15}NO$ formation, and does not change the peak at 2363 cm^{-1} for CO_2.[28] Similar analysis of the headspace of a reaction mixture that did not contain HRP fails to show the presence of either of these gases after 1 h. Nitrous oxide and carbon dioxide also did not form after 1 h upon addition of hydroxyurea to a solution of HRP in the absence of hydrogen peroxide. The addition of potassium cyanide (KCN, 50 mM) also completely suppresses the formation of these gases. Analysis after 24 h shows that the amounts of these gases do not increase over time (N_2O, 15%, CO_2, 23%). Addition of another aliquot of hydrogen peroxide after 1 h also does not produce more N_2O and CO_2.

Chemiluminescence measurements show the formation of nitrite (NO_2^-, 7%) and nitrate (NO_3^-, 19%) after 1 h of reaction of hydroxyurea with hydrogen peroxide in the presence of HRP. Again, these nitrogen oxides did not form after 1 h in the absence of HRP or hydrogen peroxide. A spectrophotometric ammonia detection assay based upon the L-glutamate hydrogenase catalyzed reductive amination of 2-oxoglutarate shows the presence of small amounts of ammonia (NH_3, 4%) from the reaction of hydroxyurea with hydrogen peroxide in the presence of HRP. All chemical yields are based upon the possibility of one molecule of hydroxyurea producing one molecule each of CO_2, NH_3, and nitrogen monoxide (NO or HNO) with nitrite and nitrate being derived from NO or HNO.

(27) Yonetani, T.; Yamamoto, H.; Erman, J. E.; Leigh, J. S.; Reed, G. H. *J. Biol. Chem.* **1972**, *247*, 2447−2455.

(28) Pinchas, S.; Laulicht, I. *Infrared Spectra of Labeled Compounds*; Academic Press: London, 1971; p 218.

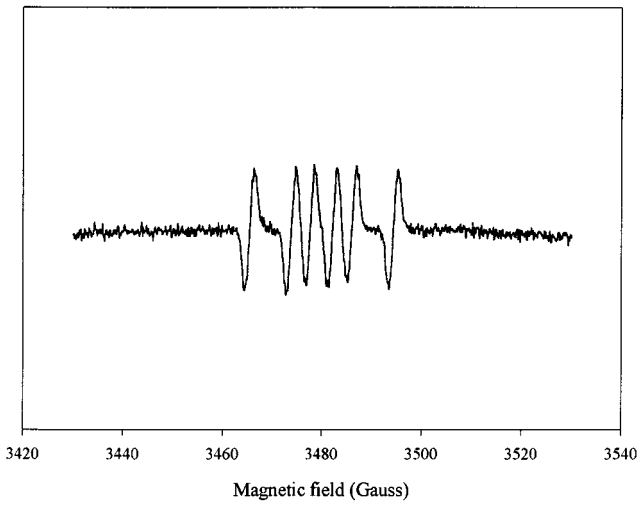
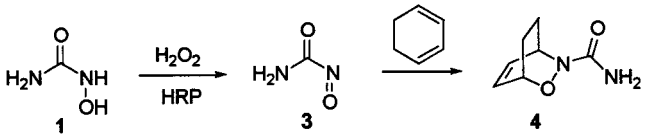


Figure 2. Room temperature EPR spectrum of the nitroxide radical (**2**) formed by the addition of hydrogen peroxide to a solution of HRP and hydroxyurea.

Scheme 1



Room temperature EPR analysis of a solution of hydroxyurea, hydrogen peroxide, and HRP reveals a distinct six-line resonance pattern characteristic of nitroxide radical (**2**, Figure 2). This spectrum is identical with reported spectra of **2** generated by the oxidation of hydroxyurea with excess hydrogen peroxide, copper(II) sulfate, or oxyHb.[9,12] This signal reaches a maximum intensity approximately 5 min after mixing the reagents and persists at least for 20 min. Addition of hydrogen peroxide to hydroxyurea in the absence of HRP does not produce a signal. The EPR spectrum at 77 K of the final reaction mixture also appears identical to an initial EPR spectrum of ferric HRP.

Addition of hydrogen peroxide to a solution of hydroxyurea and HRP in the presence of 1, 3-cyclohexadiene (50 mM) produces the cycloadduct (**4**, Scheme 1) in 42% yield after 1 h. Isolation and characterization by ^{1}H NMR spectroscopy of this cycloadduct provides clear evidence for the intermediacy of the *C*-nitroso compound (**3**) in this reaction (Scheme 1). The cycloadduct **4** did not form after 1 h in the absence of HRP or in the presence of KCN (50 mM).

Chemiluminescence NO detection shows that horseradish peroxidase directly catalyzes the formation of NO from hydroxyurea in the presence of hydrogen peroxide. Addition of small amounts of hydrogen peroxide to a degassed solution of hydroxyurea (1.0 mM) and HRP (0.2 μM) in the reaction chamber of a commercial chemiluminescence NO detector produces an immediate increase in voltage indicative of NO formation (Figure 3). A rapid burst of NO production accompanies each addition of hydrogen peroxide with the amount of NO produced being proportional to the amount of hydrogen peroxide added (Figure 3). Under these conditions, repeated additions of hydrogen peroxide generate NO. Using higher concentrations of hydrogen peroxide and hydroxyurea as above produces off-scale amounts of NO in this assay. Addition of hydrogen peroxide to hydroxyurea in the absence of HRP fails to produce any voltage change.

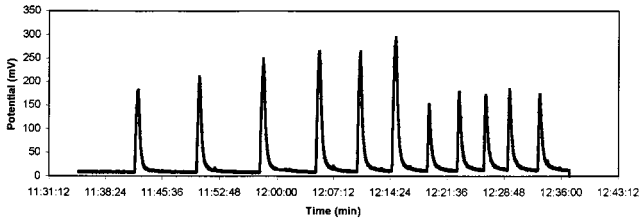


Figure 3. Chemiluminescent NO detection from the addition of hydrogen peroxide to a solution of HRP and hydroxyurea. The first six peaks arise immediately following the addition of 10 μL of a 5 mM hydrogen peroxide solution. The last five peaks arise immediately following the addition of 5 μL of a 5 mM hydrogen peroxide solution.

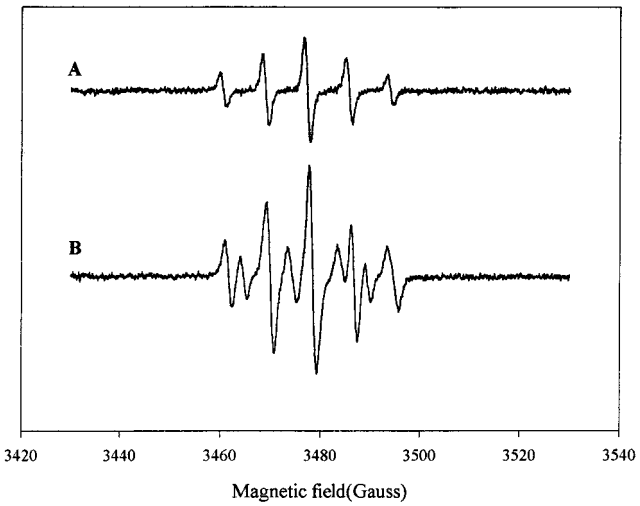
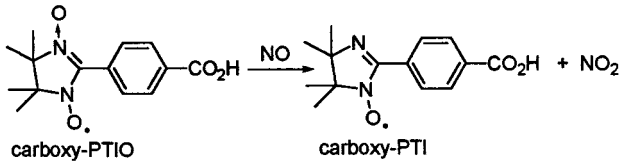


Figure 4. (A) Room temperature EPR spectrum of a mixture of HRP, hydroxyurea, and carboxy-PTIO. (B) Room temperature EPR spectrum of a mixture of HRP, hydroxyurea, carboxy-PTIO, and hydrogen peroxide.

Scheme 2



Room temperature EPR experiments with the nitric oxide selective trap, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5,-tetramethyl-1*H*-imidazolyl-1-oxy-3-oxide (carboxy-PTIO) provide further evidence of HRP catalyzed NO production from hydroxyurea in the presence of hydrogen peroxide. Carboxy-PTIO produces a five-line EPR spectrum and reacts with NO to form an imino nitroxide radical, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5,-tetramethyl-1*H*-imidazolyl-1-oxy (carboxy-PTI), which produces a distinct seven-line EPR spectrum (Scheme 2).[29] Addition of hydroxyurea to a solution of HRP and carboxy-PTIO only shows the five-line spectrum of carboxy-PTIO, indicating that hydroxyurea and HRP alone do not react to form NO (Figure 4). Addition of hydrogen peroxide to this reaction mixture rapidly produces a nine-line EPR spectrum indicative of a mixture of carboxy-PTIO and carboxy-PTI (Figure 4).[29] The formation of carboxy-PTI under these conditions demonstrates NO formation and supports the results of the chemiluminescence experiments. In the absence of hydroxyurea, the addition of hydrogen peroxide or HRP to a solution of carboxy-PTIO does not change the EPR spectra. Addition of both

(29) Akaike, T.; Maeda, H. *Methods Enzymol.* **1996**, *268*, 211−221.

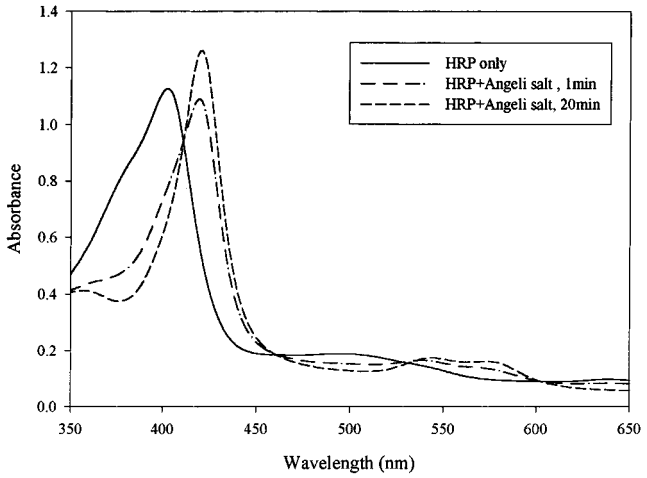


Figure 5. Absorbance spectra of ferric HRP (solid line), HRP and sodium trioxodinitrate after 1 min (long dashed line), and HRP and sodium trioxodinitrate after 20 min (short dashed line).

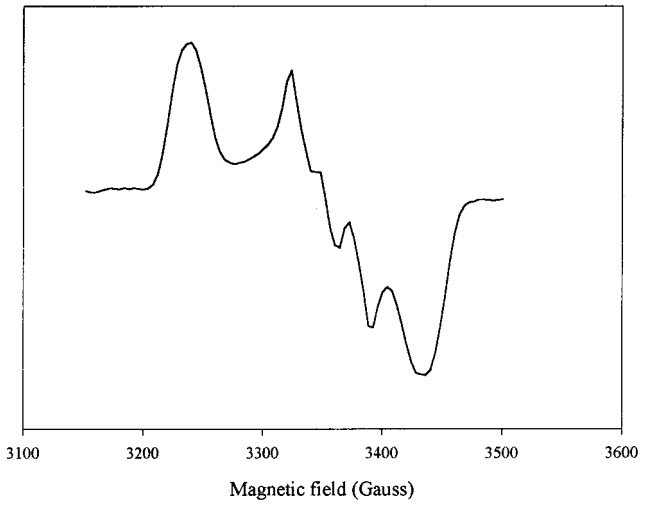


Figure 6. EPR spectrum of HRP and sodium trioxodinitrate at 77 K.

hydrogen peroxide and HRP to a solution of carboxy-PTIO in the absence of hydroxyurea rapidly quenches the five-line EPR signal.

Addition of sodium trioxodinitrate (Angeli's salt), a known donor of HNO, to an anaerobic solution of HRP produces a shift in absorbance in the Soret region from 402 to 420 nm and also an increase in absorbance at 545 and 575 nm (Figure 5). These absorbance changes correspond to the formation of a ferrous HRP−NO complex.[27] Figure 6 shows the mixture of Angeli's salt and ferric HRP produces an EPR spectrum characterized by resonances at $g = 2.08$, 2.00, and 1.95. With the exception of the well-resolved splitting of the central resonance, this spectrum appears identical with a reported spectrum of ferrous NO−HRP.[27]

Room temperature EPR experiments in the presence of carboxy-PTIO also show evidence of NO formation during the reaction of Angeli's salt with HRP (Figure 7). Addition of Angeli's salt to a solution of ferric HRP in the absence of hydrogen peroxide completely converts carboxy-PTIO to carboxy-PTI, indicating NO formation (Figure 7B). No formation of carboxy-PTI occurs upon addition of Angeli's salt to carboxy-PTIO in the absence of HRP (Figure 7A). In the presence of hydrogen peroxide, addition of Angeli's salt to ferric HRP also

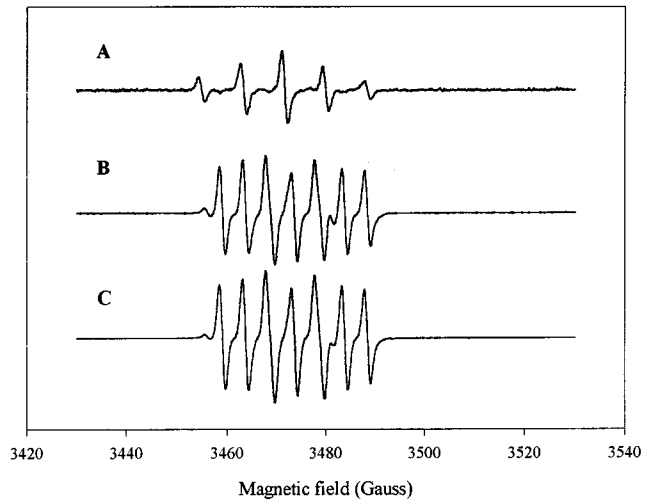


Figure 7. (A) Room temperature EPR spectrum of a mixture of sodium trioxodinitrate and carboxy-PTIO. (B) Room temperature EPR spectrum of a mixture of sodium trioxodinitrate, HRP, and carboxy-PTIO. (C) Room temperature EPR spectrum of a mixture of sodium trioxodinitrate, HRP, carboxy-PTIO, and hydrogen peroxide.

converts carboxy-PTIO to carboxy-PTI (Figure 7C). In the absence of hydrogen peroxide, the carboxy-PTI signal appears unstable and disappears within 5 min. However, in the presence of hydrogen peroxide the seven-line carboxy-PTI signal appears more stable and persists over 20 min.

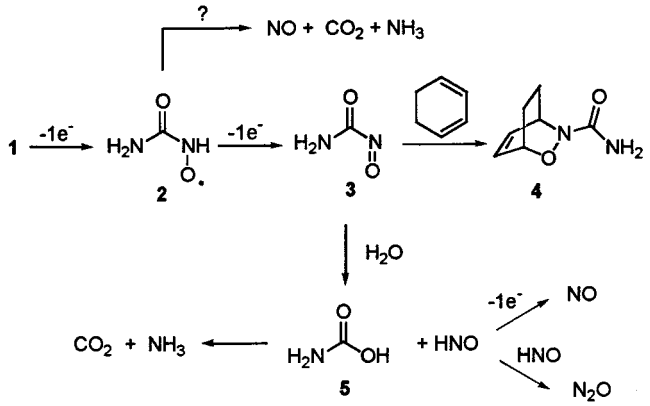
Discussion

Horseradish peroxidase catalyzes the formation of nitric oxide and nitroxyl from hydroxyurea in the presence of hydrogen peroxide as shown by chemiluminescence, EPR, and gas chromatographic analysis. Chemiluminescence NO detection provides clear and direct evidence of NO formation from this reaction and experiments with the NO-specific EPR trap, carboxy-PTIO, support these results. Gas chromatographic nitrous oxide identification provides strong evidence for the intermediacy of nitroxyl, the one-electron reduced form of NO that rapidly dimerizes and dehydrates to form N_2O.[30] The formation of nitrite and nitrate, stable oxidative decomposition products of NO,[31] further supports the intermediacy of NO and HNO in this reaction. Addition of hydrogen peroxide to hydroxyurea does not produce nitric or nitrous oxide within 1 h, indicating that the protein accelerates the production of these species. Suppression of product formation upon the addition of KCN, which binds to the ferric heme group, specifically shows the importance of this group in this reaction. These experiments also show HRP does not directly react with hydroxyurea to form nitric and nitrous oxide and hydrogen peroxide is required for product formation.

In the absence of HRP, hydrogen peroxide slowly reacts with hydroxyurea to form nitrous oxide, carbon dioxide, ammonia, and nitrite and trapping experiments also show the intermediacy of the *C*-nitroso compound (**3**) in this reaction.[21] The identification of these products and **3** during the reaction of hydroxyurea and hydrogen peroxide in the presence of HRP suggests that the HRP catalyzed reaction may be mechanistically similar to the non HRP catalyzed reaction. Scheme 3, proposed for the

(30) Hughes, M. W. *Biochim. Biophys. Acta* **1999**, *1411*, 263−272.
(31) Wink, D. A.; Darbyshire, J. F.; Nims, R. W.; Saavedra, J. E.; Ford, P. C. *Chem. Res. Toxicol*. **1993**, *6*, 23−27.

Scheme 3



reaction of hydroxyurea and hydrogen peroxide in the absence of HRP,[21] provides a possible explanation for the formation of these products during the HRP catalyzed oxidation of hydroxyurea. Single electron oxidation of hydroxyurea produces the nitroxide radical (**2**) and further single electron oxidation of **2** would produce the *C*-nitroso compound (**3**, Scheme 3). The *C*-nitroso compound (**3**) reacts with 1,3-cyclohexadiene to form cycloadduct (**4**) or rapidly hydrolyzes in the absence of 1,3-cyclohexadiene to carbamic acid (**5**) and HNO. Carbamic acid (**5**) decomposes to carbon dioxide and ammonia. Nitrous oxide forms from the rapid dimerization ($k = 1.8 \times 10^9$ M^{-1} s^{-1}) and dehydration of nitroxyl.[30] Further oxidation of HNO provides a route to nitric oxide and ultimately nitrite and nitrate. Alternatively, NO formation may arise from the direct decomposition of the nitroxide radical (**2**), as previously proposed.[10]

Scheme 3 accounts for all of the reactive intermediates and products observed in this reaction at this time. Room temperature EPR experiments and the identification of cycloadduct (**4**) from trapping experiments provide direct evidence of both the nitroxide radical (**2**) and *C*-nitroso compound (**3**) as intermediates. Chemiluminescence NO detection and EPR spectroscopy show NO formation. Gas chromatography and gas-phase infrared spectroscopy show both nitrous oxide and carbon dioxide formation. Gas-phase infrared spectroscopic analysis of the N−N bond stretching frequency indicates the formation of only $^{15}N^{15}NO$ from the reaction with ^{15}N-hydroxyurea, establishing that both nitrogen atoms of the produced N_2O derive from the NH−OH group of hydroxyurea. The formation of only $^{15}N^{15}NO$ supports the proposed mechanism of nitrous oxide formation from the dimerization of nitroxyl derived from the hydrolysis of **3** (Scheme 3). While nitrous oxide formation can be explained by nitroxyl dimerization and dehydration, other possible mechanisms such as a reaction between nitric oxide and the nitroxide radical (**2**) cannot be completely ruled out at this time. A spectrophotometric assay also indicates the production of small amounts of ammonia.

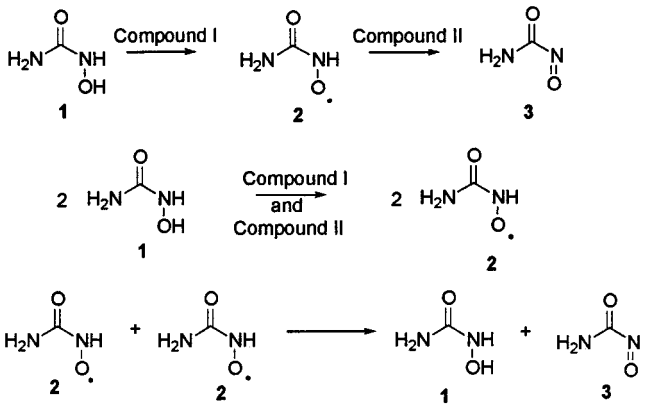
If the final stable reaction products all derive from the *C*-nitroso compound (**3**), then Scheme 3 predicts the formation of equal amounts of carbon dioxide, ammonia, and nitrogen oxides (N_2O, NO, NO_2^-, and NO_3^-). If **2** also decomposes into NO with formation of equal amounts of CO_2 and NH_3, then Scheme 3 still predicts the formation of equal amounts of CO_2, NH_3, and nitrogen oxides regardless of which reactive intermediate may be involved. The nearly equal yields of CO_2 and N_2O suggest that NO does not form from the further oxidation of HNO and possibly that NO, NO_2^-, and NO_3^- directly arise from **2**. While **2** has been proposed to decompose to NO,[10] the absence of a decomposition mechanism and complete hydroxyurea-derived product identification weakens any mechanistic interpretation based on the reactivity of **2**. Also, only about half as much CO_2 and N_2O form compared to the observed amount of cycloadduct (**4**), which serves as a measure of **3**. Such discrepancies may lie in the different sensitivities, interferences, and precision of the various analytical techniques required to measure the many reaction products. For example, the relatively high concentration of hydrogen peroxide interferes with the enzymatic NADPH-based assay and most likely accounts for the low observed ammonia yield. As Scheme 3 probably does not completely describe this complex process that includes many reactive species, strict mechanistic interpretations based on these yields at this time must remain tentative. Determination of the product ratios under initial reaction conditions may provide useful mechanistic information.

While product formation occurs rapidly within the first hour of the HRP catalyzed reaction in the presence of excess hydrogen peroxide, little change in the amount of products occurs over longer periods of time. Under these conditions, further addition of hydrogen peroxide does not generate more products suggesting a loss of enzyme activity. Similarly, in the presence of excess hydrogen peroxide HRP catalyzes the formation of NO and L-citrulline from L-*N*-hydroxyarginine, the biochemical precursor of NO.[32] These products initially form rapidly but the activity of the system decreases with time.[32] Denaturing effects of NO or other nitrogen oxides possibly reduce the activity of this system and absorption spectroscopy shows evidence for a ferric HRP−NO complex.[32] Absorption studies show no evidence for the formation of a ferric HRP−NO complex during the HRP catalyzed oxidation of hydroxyurea, suggesting that the activity loss in this system may result from oxidative damage to HRP by the excess hydrogen peroxide. Alternatively, a catalytically inactive ferric HRP−NO complex could form but not be observable as nitric oxide reversibly binds to the ferric heme of HRP and may not be stable under the aerobic conditions used in this study.[27] Also, hydrogen peroxide, used in excess in this work, reverses NO binding to ferric HRP.[33] The addition of small amounts of hydrogen peroxide to a mixture of HRP and hydroxyurea repeatedly produces NO without the apparent loss of activity as shown by chemiluminescence studies. Such conditions may be useful for further mechanistic studies.

Scheme 3 requires a one-electron oxidant for the conversion of hydroxyurea to the nitroxide radical (**2**) and for the conversion of **2** to the *C*-nitroso compound (**3**). The lack of product formation from the reaction of HRP and hydroxyurea in the absence of hydrogen peroxide indicates that the ferric heme group of HRP does not oxidize hydroxyurea. This result contrasts the ability of methemoglobin to react with hydroxyurea in the absence of hydrogen peroxide to produce iron nitrosyl hemoglobin.[23] In the classic peroxidase activation and reaction scheme, hydrogen peroxide formally performs a two-electron oxidation of HRP to give a reactive intermediate designated compound I.[34] Compound I removes a single electron from a

(32) Boucher, J. L.; Genet, A.; Vadon, S.; Delaforge, M.; Mansuy, D. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1158−1164.
(33) Ischiropoulos, H.; Nelson, J.; Duran, D.; Al-Mehdi, A. *Free Radical Biol. Med.* **1996**, *20*, 373−381.
(34) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman and Company: New York, 1979; pp 488−493.

Scheme 4



substrate to form a second reactive intermediate termed compound II.[34] Finally, compound II oxidizes a second substrate molecule to return the enzyme to its resting state.[34] Compounds I and II thus provide potential oxidants for the conversion of hydroxyurea to **2** and **3** depicted in Scheme 3. Hydroxyurea could be oxidized by compound I to form **2**, which could further be oxidized by compound II to produce **3** and return the enzyme to the resting state (Scheme 4, first reaction). Such a sequence represents an overall two-electron oxidation of hydroxyurea by HRP. Alternatively, compounds I and II could each oxidize a single hydroxyurea molecule to **2** and these radicals could disproportionate to form hydroxyurea and **3** (Scheme 4, second and third reactions).

The rapid shift in the maximum absorbance in the Soret region from 402 to 419 nm followed by a slower return to 402 nm indicates the accumulation of compound II. Such results suggest that compound I, generated by the hydrogen peroxide oxidation of ferric HRP, rapidly oxidizes hydroxyurea to form the nitroxide radical (**2**) and compound II. Compound II then oxidizes hydroxyurea or **2** in a slower rate-determining step. This observation supports the general trend that substrates react faster with HRP compound I than compound II.[35] Addition of excess hydrogen peroxide to ferric HRP in the absence of hydroxyurea shows complete conversion to HRP compound II as judged by absorption spectroscopy and noted for other peroxidases.[36,37] The return of the absorption spectrum of compound II to resting enzyme upon addition of hydroxyurea clearly shows that compound II can oxidize hydroxyurea to presumably form radical (**2**). These results favor a mechanism where both compounds I and II oxidize hydroxyurea to **2**, which then disproportionates to **3** and hydroxyurea (Scheme 4, second and third reactions). Whether compound II can oxidize **2** remains unclear and sequential one-electron oxidations of hydroxyurea by compounds I and II to form **3** cannot be ruled out at this time (Scheme 4, first reaction). However, the identification of compound II in this process eliminates a direct two-electron oxidation of hydroxyurea by compound I as a possible mechanism for the formation of **3**.

The inclusion of compounds I and II as intermediates in the oxidation of hydroxyurea by hydrogen peroxide may explain the relatively slow product formation observed in the non-HRP catalyzed reaction. As hydrogen peroxide does not act as a one-electron oxidant, adventitious metals were suggested to play a role in product formation.[21] In support of the role of adventitious metals in the non-HRP catalyzed reaction, our EPR results show that the nitroxide radical (**2**) does not form upon addition of hydrogen peroxide to hydroxyurea in buffer prepared from distilled and deionized water. In contrast, this same experiment performed in buffer prepared from tap water rapidly showed the formation of the nitroxide radical (**2**, data not shown). Also, addition of a small amount (10 mol %) of copper(II) sulfate to a mixture of hydroxyurea and hydrogen peroxide produces large amounts of nitrous oxide (46%) within 1 h. Such results suggest that compounds I and II catalyze the hydroxyurea reaction by acting as the one-electron oxidants required for ultimate product formation as shown in Scheme 3.

While previous work shows that NO binds to both ferrous and ferric HRP to form stable complexes,[27] recent work shows that NO acts as a substrate for both compound I and compound II of HRP.[38] Nitric oxide also acts as a substrate for a number of mammalian peroxidases under physiological conditions.[37] Related work demonstrates that NO modulates the catalytic activity of myeloperoxidase primarily through the formation of ferrous and ferric iron heme NO complexes.[39] Such results indicate that NO formed in the HRP catalyzed oxidation of hydroxyurea could potentially compete with hydroxyurea as a substrate for compound I or II. Single electron oxidation of NO would produce NO^+, which would form nitrite upon hydration. At the present, no direct evidence exists to indicate that NO acts as a substrate of HRP during the oxidation of hydroxyurea.

Identification of nitrous oxide during the HRP catalyzed oxidation of hydroxyurea provides evidence for the intermediacy of nitroxyl (HNO). The oxidation of HNO provides an alternative route to decomposition of **2** for NO production (Scheme 3). Ferric heme containing proteins including methemoglobin and metmyoglobin oxidize nitroxyl to form NO and ferrous heme, which combine to give a stable iron nitrosyl complex.[40,41] Alternatively, nitric oxide could form from the oxidation of nitroxyl directly by the nitroxide radical (**2**) to also form hydroxyurea (**1**). Nitroxyl reacts with oxygen to produce reactive nitrogen oxide species and any such reactions would compete with nitric oxide formation and must be considered.[42]

While hydroxyurea did not react with ferric HRP, absorption spectroscopy shows that ferric HRP oxidizes nitroxyl generated from Angeli's salt to produce a ferrous HRP−NO complex. The conversion of carboxy-PTIO to carboxy-PTI in the presence of Angeli's salt and HRP indicates that this system also generates NO. The conversion of carboxy-PTIO to carboxy-PTI under these conditions requires the presence of free nitric oxide and suggests the reaction of HNO and ferric HRP occurs through an outer sphere mechanism similar to the reaction of HNO with ferricytochrome C.[40] Thermodynamics also favor the oxidation of HNO to NO ($E_{NO/NO^-}{}^\circ = -0.33$ V) over the oxidation of NO to NO^+ ($E_{NO^+/NO}{}^\circ = +1.2$ V) and the ability of HRP compounds I and II to oxidize NO suggests that these species

(35) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1976**, *19*, 187−251.
(36) Marquez, L. A.; Dunford, H. B.; Van Wart, H. *J. Biol. Chem.* **1990**, *265*, 5666−5670.
(37) Abu-Soud, H. M.; Hazen, S. L. *J. Biol. Chem.* **2000**, *275*, 37524−37532.
(38) Glover, R. E.; Koshkin, V.; Dunford, H. B.; Mason, R. P. *Nitric Oxide: Biol. Chem.* **1999**, *6*, 439−444.
(39) Abu-Soud, H. M.; Hazen, S. L. *J. Biol. Chem.* **2000**, *275*, 5425−5430.
(40) Doyle, M. P.; Mahapatro, S. N.; Broene, R. D.; Guy, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 593−599.
(41) Bazylinksi, D. A.; Hollocher, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 7982−7986.
(42) Miranda, K. M.; Espey, M. G.; Yamada, K.; Krishna, M.; Ludwick, N.; Kim, S.; Jourd'heuil, D.; Grisham, M. B.; Feelisch, M.; Fukuto, J. M.; Wink, D. A. *J. Biol. Chem.* **2001**, *276*, 1720−1727.

should be capable of oxidizing HNO to NO.[43] EPR experiments with carboxy-PTIO also show NO formation upon addition of Angeli's salt to ferric HRP in the presence of hydrogen peroxide suggesting that compounds I and II can mediate the conversion of HNO to NO. While these experiments show NO formation from HRP and Angeli's salt, it is not clear at this time whether HRP mediated oxidation of HNO occurs during the hydroxyurea reaction.

With use of horseradish peroxidase as a model, these results show in vitro peroxidase catalyzed NO and HNO formation from the clinically used drug hydroxyurea. Peroxidase-catalyzed NO release from hydroxyurea could play an important role in the beneficial effects of hydroxyurea for the treatment of various conditions. Hydroxyurea-derived NO should inhibit ribonucleotide reductase thus blocking DNA synthesis. The beneficial effects of NO in sickle cell disease remain controversial but could include direct vasodilation, inhibition of platelet aggregation, or the modification of hemoglobin. Partial iron nitrosylation may form hemoglobins with improved solubility properties that retain the ability to function as oxygen carriers capable of oxygen delivery under physiological conditions.[19,44] *S*-Nitrosohemoglobin may form through an oxygen-dependent NO migration from the iron atom of HbNO to the thiol group of the β-93 cysteine residue.[15,16] In addition to the important roles that *S*-nitrosohemoglobin plays in blood pressure control by releasing NO at low oxygen pressures,[45,46] *S*-nitrosation of the β-93 residue favors the high affinity *R*-state, which does not polymerize, and increases the oxygen affinity of the protein.[15,47,48]

EPR studies already demonstrate the in vivo formation of HbNO in the blood of both rats and humans upon administration of hydroxyurea and ^{15}N-labeling studies shows that the NO derives from hydroxyurea.[13,14] While hydroxyurea directly reacts with oxy, deoxy, and metHb to form HbNO, these reactions occur slowly in vitro and may not be responsible for in vivo HbNO formation.[22,23,49] Other oxidative enzymes have been suggested as being responsible for catalyzing the metabolism of hydroxyurea to NO, which could then react with deoxyHb to form HbNO.[13,14] This work shows in vitro peroxidase-catalyzed NO and HNO production from hydroxyurea and suggests the possibility of in vivo NO release especially in cells that contain significant amounts of peroxidases and hydrogen peroxide, such as neutrophils and monocytes. Also, oxidation of hydroxyurea by the peroxidase-like ferryl hemoglobin, formed by the reaction of hydrogen peroxide and metHb, provides another interesting potential route of NO formation supported by this work. Indeed, ferryl myoglobin converts the structurally similar hydroxylamine to nitric oxide.[50] Finally, these results reinforce the recent work indicating that hydroxyurea releases NO and HNO upon oxidation and warrant the consideration of such species in the discussion of the biological properties of hydroxyurea.

Summary

These studies demonstrate that horseradish peroxidase (HRP) catalyzes the formation of nitric oxide (NO) from the clinically used drug hydroxyurea. The reactive HRP intermediate compounds I and II serve as one-electron oxidants of hydroxyurea to produce a nitroxide radical (**2**) and a *C*-nitroso species (**3**), which decompose to NO and nitroxyl (HNO), the one-electron-reduced form of NO. These results indicate that peroxidase-catalyzed NO release from hydroxyurea could play an important role in the overall pharmacological properties of this drug.

Acknowledgment. This work was supported by the National Institutes of Health (HL62198, S.B.K.), the American Heart Association (B98423N, D.B.K.S., 963031N, S.B.K.) and a Henry Dreyfus Teacher Scholar Award (S.B.K.). The NMR spectrometers used in this work were purchased with partial support from the National Science Foundation (CHE-9708077) and the North Carolina Biotechnology Center (9703-IDG-1007). The authors wish to thank Ms. Challice Bonifant, Dr. Yueping Xu, and Dr. Roy Ware for performing some preliminary experiments and technical assistance.

JA012271V

(43) Wink, D. A.; Feelisch M. In *Methods in Nitric Oxide Research*; Feelisch, M., Stamler, J. S., Eds.; John Wiley and Sons: Chichester, 1996; pp 403−413.
(44) Yonetani, T.; Tsuneshige, A.; Zhou, Y.; Chen, X. S. *J. Biol. Chem.* **1998**, *273*, 20323−20333.
(45) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J. S. *Nature* **1996**, *380*, 221−226.
(46) Pawloski, J. R.; Hess, D. T.; Stamler, J. S. *Nature* **2001**, *409*, 622−626.
(47) Patel, R. P.; Hogg, N.; Spencer, N. Y.; Kalyanaraman, B.; Matalon, S.; Darley-Usmar, V. M. *J. Biol. Chem.* **1999**, *274*, 15487−15492.
(48) Bonaventura, C.; Ferruzzi, G.; Tesh, S.; Stevens, R. D. *J. Biol. Chem.* **1999**, *274*, 24742−24748.
(49) Kim-Shapiro, D. B.; King, S. B.; Bonifant, C. L.; Kolibash, C. P.; Ballas, S. K. *Biochim. Biophys. Acta* **1998**, *1380*, 64−74.
(50) Taira, J.; Misik, V.; Riesz, P. *Biochim. Biophys. Acta* **1997**, *1336*, 502−508.