Articles

Catalase-Mediated Nitric Oxide Formation from Hydroxyurea

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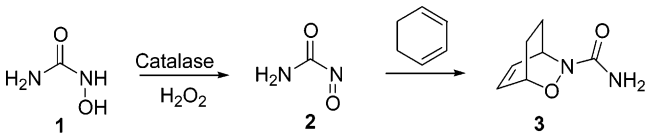
Hydroxyurea reduces the incidence of painful crises in patients with sickle cell disease and has recently been approved for the treatment of this condition. A number of in vitro studies show that the oxidation of hydroxyurea results in the formation of nitric oxide, which also has drawn considerable interest as a sickle cell disease therapy. While patients on hydroxyurea demonstrate elevated levels of nitric oxide-derived metabolites, little information regarding the site or mechanism of the in vivo conversion of hydroxyurea to nitric oxide exists. Chemiluminescence detection experiments show the ability of catalase to catalyze the formation of nitrite and nitrate from hydroxyurea. Spectroscopic studies show that the reaction of hydroxyurea and catalase in the presence of a hydrogen peroxide generating system produces a ferrous−NO catalase complex. Trapping studies indicate the intermediacy of a nitroso species during this reaction. The proposed mechanism for this conversion includes initial hydrogen peroxide-dependent oxidation of hydroxyurea by catalase to form the nitroso species, hydrolysis of this nitroso species to produce nitroxyl, and reductive nitrosylation of the ferric heme of catalase by nitroxyl to yield the ferrous−NO catalase complex. Addition of Angeli's salt, a nitroxyl donor, to ferric catalase also produces the ferrous−NO catalase complex. Spectroscopic studies show that the ferrous−NO catalase complex releases nitric oxide as judged by the oxyhemoglobin assay and an NO specific EPR specific trap. These results demonstrate nitric oxide production from the ferric catalase oxidation of nitroxyl and identify a catalase-mediated pathway as a potential source of nitric oxide production from hydroxyurea.

Introduction

Hydroxyurea (**1**, Scheme 1) finds use in the treatment of various cancers and sickle cell disease.[1−3] Hydroxyurea reduces the incidence of painful crises in patients with sickle cell disease by increasing the level of fetal hemoglobin (HbF), a genetically distinct hemoglobin that inhibits polymerization of sickle cell hemoglobin (HbS).[2−4] The ability of hydroxyurea to increase HbF levels varies among different patients, suggesting that hydroxyurea may also benefit patients through other mechanisms, including alteration of red cell−endothelial cell interactions and improved red cell rheological properties.[4] Hydroxyurea also acts as a source of NO, which plays an important role in the maintenance of normal blood pressure and flow, and has drawn considerable interest as a sickle cell disease treatment.[5−7]

Sickle cell patients demonstrate in vivo NO formation during hydroxyurea therapy. These patients show significant increases in plasma and red cell nitrite, nitrate, and iron nitrosyl hemoglobin (HbNO) levels one to 2 h after administration of an average dose of 15 mg/kg of hydroxyurea.[8] Time course studies reveal that these NO-derived metabolites increase up to 2 h and then begin to decline.[8] Another study on sickle cell disease patients taking hydroxyurea shows similar trends regarding plasma nitrite/nitrate and an increase in plasma cyclic guanylate monophosphate (cGMP).[9] As NO activates soluble guanylate cyclase (sGC), the enzyme that catalyzes the cyclization of guanylate triphosphate (GTP) to cGMP, increased cGMP levels imply an increase in NO.[9] In addition, other experiments using both erythroleukemic cells and primary human erythroblasts link HbF gene expression to a sGC-cGMP-dependent protein kinase pathway.[10] Recent in vitro work shows that hydroxyurea and two other mechanistically different NO donors increase γ-globin gene expression and fetal hemoglobin levels in human erythroid progenitor cells by a sGC dependent pathway.[11] Together, these studies strongly suggest that hydroxyurea stimulates HbF synthesis by increasing cGMP levels through metabolism to NO and subsequent sGC activation.

Scheme 1



Despite these results, little detailed information regarding the site or mechanism of in vivo NO production from hydroxyurea exists. Incubation of human blood (oxygenated, deoxygenated, or cycled between oxygenated and deoxygenated) with hydroxyurea (10 mM) does not produce a detectable amount of HbNO after 2 h as determined by electron paramagnetic

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Table 1. Nitrite/Nitrate Production from Hydroxyurea (1 mM) and Catalase after Incubation at 37 °C for 3 h

reaction system	NO_2^-/NO_3^- (μM)
hydroxyurea	3.8 ± 0.2
hydroxyurea/Cat	4.2 ± 0.4
hydroxyurea/Cat/glucose/glucose oxidase	120.2 ± 8.5
hydroxyurea/glucose/glucose oxidase	4.7 ± 0.2
hydroxyurea/Cat(heat inact.)/glucose/glucose oxidase	5.8 ± 0.7
hydroxyurea/Cat/glucose/glucose oxidase/3-AT	8.3 ± 0.7

resonance (EPR) spectroscopy.[12] Similarly, the NO producing reactions of hydroxyurea and oxy and deoxy hemoglobin (Hb) occur at only modest rates that do not kinetically support the observed increases in NO metabolites that occur within short periods of time in patients on hydroxyurea therapy.[13−15] As such, alternative mechanisms of in vivo NO release from hydroxyurea rather than the direct reaction with hemoglobin or other blood components must be considered.

Previous studies show the reaction of catalase with hydroxylamine yields a catalase−NO complex, which competently activates sGC.[16,17] We find that reaction of catalase with the structurally related hydroxyurea rapidly forms nitrite, nitrate, and a ferrous catalase−NO complex. More specifically, catalase appears to metabolize hydroxyurea to nitroxyl (HNO), the one-electron reduced form of nitric oxide, which reduces catalase to give the ferrous iron−NO catalase complex. This complex releases NO that reacts with oxyHb and an NO-specific EPR trap. These results thus describe a potential pathway of NO formation from hydroxyurea that possibly explains in vivo HbNO production and HbF stimulation in patients taking hydroxyurea.

Results

Chemiluminescence NO analysis following reaction mixture reduction with a refluxing vanadium(III) chloride/HCl solution provides a rapid and accurate measurement of the stable oxidative decomposition products of NO, nitrite, and nitrate (NO_2^-/NO_3^-).[18] Table 1 summarizes the production of NO_2^-/NO_3^- from the reaction of catalase (Cat) and hydroxyurea. Incubation of hydroxyurea (1 mM) alone with purified Cat (1.0 μM, bovine liver) fails to produce a significant amount of NO_2^-/NO_3^-, showing that Cat and hydroxyurea do not react (Table 1). Addition of glucose (10 mM) and glucose oxidase (7.5 units), a slow hydrogen peroxide-generating system,[19] to the same mixture of hydroxyurea and catalase increases the level of NO_2^-/NO_3^- 28.6-fold (Table 1). Chemiluminescence NO analysis following reaction mixture reduction with potassium iodide in acetic acid, which only reduces nitrite to NO, shows that nitrate (113.0 ± 8.0 μM) constitutes the major species of this mixture. Incubation of this system with 3-amino-1,2,4 triazole (3-AT), a known Cat inhibitor,[20] or using heat-deactivated Cat abolishes the increase in NO_2^-/NO_3^- (Table 1). In addition, incubation of hydroxyurea with glucose/glucose oxidase in the absence of catalase fails to result in the accumulation of NO_2^-/NO_3^- (Table 1).

Absorption measurements show the characteristic Soret absorbance for ferric catalase at 405 nm shifts to 412 nm during the reaction of hydroxyurea and catalase in the presence of glucose and glucose oxidase under air (Figure 1). An increase in absorbance at 545 and 575 nm also occurs during this reaction (Figure 1). Over time, the absorbance spectrum of the reaction mixture returns to that of resting catalase. No changes occur in the absorption spectrum of Cat upon addition of hydroxyurea in the absence of glucose/glucose oxidase. The absorption spectrum of the reaction mixture appears different from the known spectrum of the ferric catalase−NO complex,[21] which shows a characteristic Soret absorbance at 427 nm and prominent peaks at 540 and 576 nm (Figure 1).

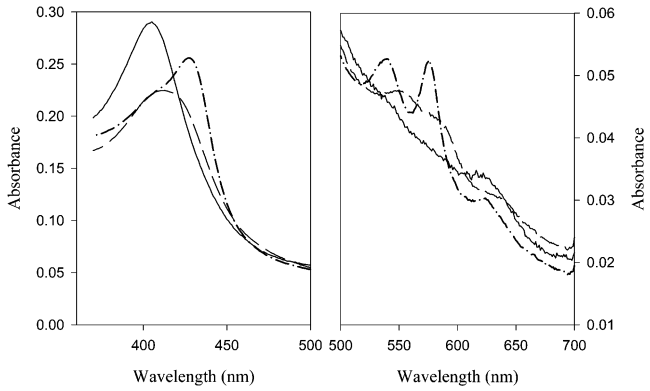


Figure 1. Absorbance spectra of ferric catalase (2 μM, solid line), the aerobic reaction of catalase (2 μM) and hydroxyurea (50 mM) in the presence of glucose (10 mM) and glucose oxidase (15 units) in phosphate buffer (100 mM, pH 7.4) at room temperature (long dash line), and the reaction of catalase (2 μM) and excess nitric oxide in phosphate buffer (100 mM, pH 7.4) at room temperature (dash-dot line). The left panel shows absorbance in the Soret region and the right panel shows absorbance between 500 and 700 nm.

The EPR spectrum of ferric catalase shows three resonances: a broad resonance at $g = 6.26$, a resonance at $g = 4.28$, and a very weak resonance at $g = 1.98$ (Supporting Information). In general, this spectra appears similar to a previously reported EPR spectra of human erythrocyte catalase.[22] The EPR spectrum from the reaction of hydroxyurea with catalase in the presence of glucose/glucose oxidase at 130 K shows the appearance of a new prominent peak ($g = 1.99$), characteristic of a ferrous heme−NO complex (Supporting Information).[23,24] This spectrum also indicates the presence of ferric catalase in the reaction mixture. Expansion of this resonance reveals a three peak pattern with a hyperfine constant of 17.4 G consistent with electronic coupling to ^{14}N (Figure 2). This spectra appears similar to the previously reported spectra of the catalase−NO complex formed during the reaction of catalase and hydroxylamine.[17] The EPR spectrum of this reaction mixture also differs from the spectrum of the addition of NO to ferric catalase (Supporting Information). No changes occur in the EPR spectrum of Cat upon addition of hydroxyurea in the absence of glucose/glucose oxidase. Room temperature EPR spectroscopy fails to indicate the intermediacy of the previously described hydroxyurea-derived nitroxide radical.[25]

Gas chromatographic analysis of the headspace above the reaction mixture of hydroxyurea and catalase in the presence of glucose and glucose oxidase reveals the presence of carbon dioxide (CO_2). These measurements fail to provide any evidence of nitrous oxide (N_2O) formation during this reaction. Addition of glucose and

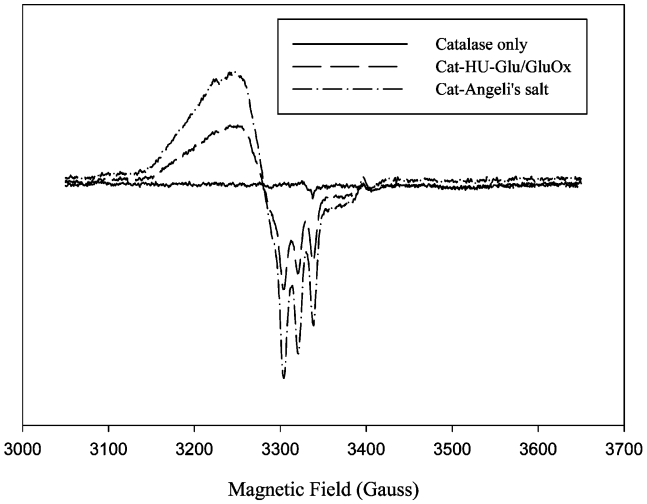


Figure 2. Expanded EPR spectra at 130 K of ferric catalase (solid line), the reaction of catalase and hydroxyurea in the presence of glucose and glucose oxidase (long dash line), and the anaerobic reaction of catalase (0.8 mM) and Angeli's salt (0.8 mM) in phosphate buffer (100 mM, pH 7.4) at room temperature (dash-dot line).

glucose oxidase to a solution of hydroxyurea and catalase in the presence of 1, 3-cyclohexadiene (50 mM) produces the cycloadduct (**3**, Scheme 1) after 3 h. Isolation and characterization by ^{1}H NMR spectroscopy of this cycloadduct provides clear evidence for the intermediacy of the *C*-nitroso compound (**2**) in this reaction (Scheme 1).

Absorption measurements reveal the rapid conversion of oxyHb to metHb during the reaction of catalase and hydroxyurea in the presence of glucose and glucose oxidase (Supporting Information). The shift in the Soret absorbance from 416 to 405 nm and the increases in absorbance at 500 and 635 nm and the decreases in absorbance at 540 and 577 nm demonstrate the conversion of oxyHbA to metHbA. Analysis of the change in absorbance over time reveals an apparent rate constant for this process of $k = 2.37 \times 10^{-2} \pm 1.38 \times 10^{-3}$ min^{-1}. Under the same conditions, the direct reaction of hydroxyurea with oxyHb to produce metHb does not occur fast enough ($k = 7.54 \times 10^{-4} \pm 2.10 \times 10^{-5}$ min^{-1})[26] to account for the rapid observed rate of conversion. Appreciable amounts of metHb also do not form in the absence of hydroxyurea.

Treatment of catalase with the HNO donor, sodium trioxodinitrate (Angeli's salt), under anaerobic conditions should provide a method for the preparation of the ferrous−NO catalase complex. Absorption measurements show the Soret absorbance for ferric catalase at 405 nm gradually shifts to 421 nm upon addition of Angeli's salt (Figure 3). Under anaerobic conditions, this spectrum does not change over time. Increases in absorbance at 555 and 580 nm also occur after the addition of Angeli's salt to ferric catalase (Figure 3). While this spectrum differs from the spectrum of the reaction of hydroxyurea with catalase in the presence of glucose and glucose oxidase (Figure 1), the addition of Angeli's salt to this reaction mixture produces an absorption spectrum nearly identical to the spectrum from the reaction of catalase and Angeli's salt (Figure 3). The absorption spectrum of the reaction of catalase and Angeli's salt also differs from the spectrum of the ferric catalase−NO complex (Figures 1 and 3).[21] Fitting the spectrum from the reaction of hydroxyurea and catalase to the spectra of ferric catalase and the ferrous−NO catalase complex reveals the reaction mixture consists of 25% ferric catalase and 75% of the ferrous−NO catalase complex (Figure 3). EPR spectroscopy shows that treatment of catalase with Angeli's salt produces a spectrum nearly identical to the EPR spectrum from the reaction of hydroxyurea and catalase with a prominent three-line absorbance at $g = 1.99$ ($a = 17.4$) (Figure 2).

Room-temperature EPR experiments using the NO selective trap, carboxy-PTIO, provide further evidence of NO release from the ferrous−NO catalase complex. Carboxy-PTIO produces a five-line EPR spectrum and reacts with NO to form an imino nitroxide radical, 2-(4-

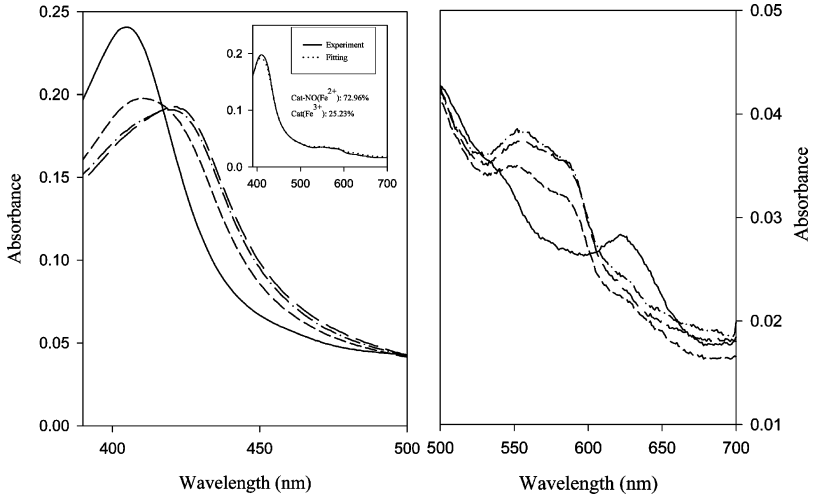


Figure 3. Absorbance spectra of ferric catalase (solid line), the aerobic reaction of catalase (2 μM) and hydroxyurea (50 mM) in the presence of glucose (10 mM) and glucose oxidase (15 units) in phosphate buffer (100 mM, pH 7.4) at room temperature (short dash line), the anaerobic reaction of ferric catalase (2 μM) and Angeli's salt (20 μM) in phosphate buffer (100 mM, pH 7.4) at room temperature (dash-dot line), and the addition of Angeli's salt to the hydroxyurea/catalase reaction mixture in the presence of glucose and glucose oxidase (long dash line). The left panel shows absorbance in the Soret region and the right panel shows absorbance between 500 and 700 nm. Inset shows the spectrum from the reaction of hydroxyurea with catalase fit to the spectra of ferric catalase and the ferrous−NO catalase complex.

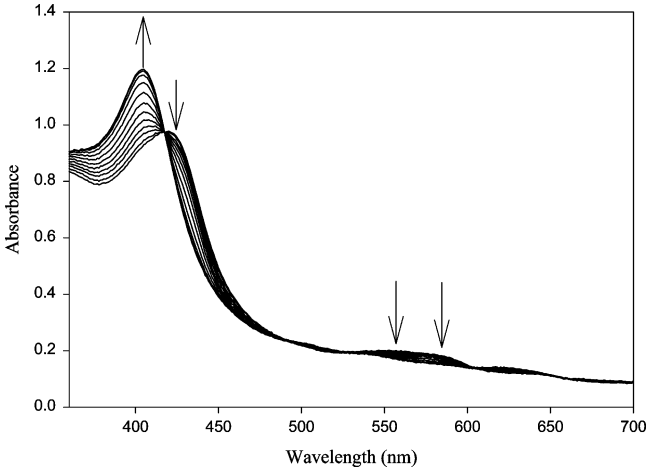


Figure 4. Absorption spectrum of the exposure of the ferrous−NO catalase complex (10 μM) in phosphate buffer (100 mM, pH 7.4) at room temperature to air. UV−vis measurements were taken every 5 min. Arrows indicate increasing and decreasing absorbance.

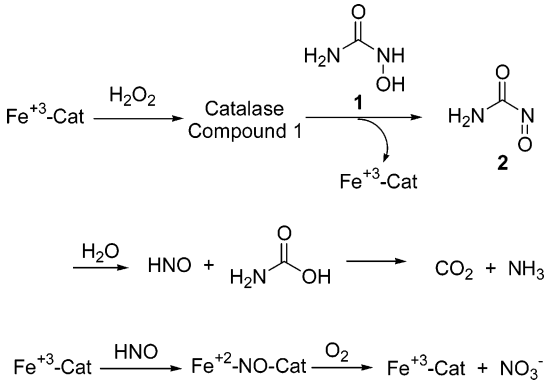
carboxyphenyl)-4,5-dihydro-4,4,5,5,-tetramethyl-1*H*-imidazolyl-1-oxy (carboxy-PTI), which produces a distinct seven-line EPR spectrum.[27] Addition of carboxy-PTIO to a solution of the ferrous−NO catalase complex generated by the anaerobic incubation of ferric catalase with Angeli's salt shows the seven-line spectrum of carboxy-PTI indicating NO transfer from the ferrous−NO catalase complex to the trap (Supporting Information). The seven-line carboxy-PTI signal appears stable and persists for over 20 min. The addition of Angeli's salt to carboxy-PTIO in the absence of catalase does not produce carboxy-PTI.

Exposure of the ferrous−NO catalase complex to air results in the formation of ferric catalase as shown by UV−vis spectroscopy (Figure 4). These measurements show the Soret absorbance shifts from 421 to 405 nm of ferric catalase and the decrease of the pronounced absorbances at 555 and 580 nm (Figure 4). This process also demonstrates a clean isobestic point at 417 nm indicating the presence of only two species. Analysis of these absorbance changes over time yields an apparent rate constant for this reaction of 5.03×10^{-4} s^{-1}. Chemiluminescence detection measurements (after subtraction of the nitrite produced during the decomposition of Angeli's salt) reveal that the aerobic decomposition of the ferrous−NO catalase complex (10 μM) yields a similar amount of nitrate (8.0 ± 1.3 μM, $n = 3$).

Discussion

Chemiluminescence experiments reveal that catalase in the presence of a hydrogen peroxide generating system converts hydroxyurea to nitrite and nitrate and provide evidence for the intermediacy of NO. These NO-derived metabolites form from a physiologically relevant concentration of hydroxyurea (1 mM) and within 3 h, a time scale compatible with the observed increase in nitrite/nitrate in patients undergoing hydroxyurea therapy.[8] Nitrite/nitrate could also potentially form from the reaction of nitroxyl with oxygen. Further experiments with the inhibitor 3-AT and heat-inactivated catalase demonstrate the requirement of catalase for nitrite/nitrate formation from hydroxyurea.

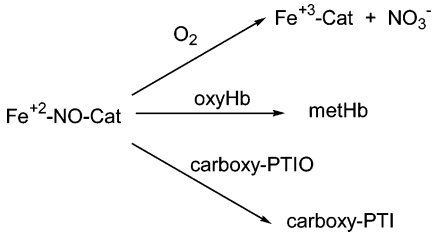
Scheme 2



EPR spectroscopy shows that under these conditions the reaction of hydroxyurea and catalase produces a mixture of ferric catalase and a ferrous−NO catalase complex. The appearance of the characteristic triplet signal at $g = 1.99$ in the EPR spectra provides strong evidence for a ferrous−NO catalase complex.[23,24] Both EPR and absorption spectroscopy confirm that this complex differs from the ferric−NO catalase complex formed by the direct addition of NO to ferric catalase.[21,28] Also, the EPR spectrum from the mixture of the HNO donor Angeli's salt with ferric catalase appears nearly identical to the reaction spectrum at $g = 1.99$. This result provides further support for the identity of the ferrous−NO complex as HNO reductively nitrosylates ferric heme proteins to produce the ferrous−NO complexes.[25,29−32] While the absorption spectra from the reaction of hydroxyurea and catalase shows some differences compared to the spectra from the reaction of Angeli's salt and ferric catalase, the addition of Angeli's salt to the hydroxyurea reaction mixture produces an absorption spectra nearly identical to the spectra from the direct addition of Angeli's salt to ferric catalase. This change suggests that the reaction mixture consists of ferrous−NO catalase and ferric catalase, which reacts with HNO to form the ferrous−NO complex. The addition of less than stoichiometric amounts of Angeli's salt to ferric catalase also produces absorption spectra which appear similar to the hydroxyurea and ferric catalase reaction spectrum indicating a mixture of ferric catalase and the ferrous−NO complex. The similarity of the absorption spectrum from the reaction with the fitted spectrum generated from the spectra of ferric catalase and the ferrous−NO catalase complex further supports the assertion that the reaction mixture contains these two components. While these results differ from a recent report that states no reaction occurs between HNO generated from Angeli's salt and catalase,[33] in our hands the mixture of anaerobic solutions of Angeli's salt and ferric catalase reproducibly yields the ferrous−NO complex of catalase. As dithionite generally fails to reduce the ferric iron of catalase,[21,34] the reductive nitrosylation of ferric catalase with HNO (from Angeli's salt) provides a convenient method for the preparation of the ferrous−NO catalase complex.

Scheme 2 depicts the proposed mechanism for the formation of a ferrous−NO catalase complex during the reaction with hydroxyurea. Hydrogen peroxide generated from the glucose/glucose oxidase reaction activates ferric catalase to Compound I. Direct two-electron oxidation of hydroxyurea by the catalase Compound I

Scheme 3



species generates the *C*-nitrosoformamide (**2**) and trapping studies with 1,3-cyclohexadiene support the intermediacy of **2**. Such a conversion finds precedence in the catalase-mediated two-electron oxidation of a variety of substrates including ethanol.[35] The oxidation of *N*-hydroxycyanamide to nitrosyl cyanide by catalase is particularly similar to the oxidation of hydroxyurea to the *C*-nitrosoformamide (**2**, Scheme 2).[36] Unlike the reaction of hydroxyurea and horseradish peroxidase,[25] the failure of room temperature EPR measurements to identify the nitroxide radical of hydroxyurea argues against single electron oxidation (or H atom abstraction) processes. However, the failure to detect the nitroxide radical does not necessarily preclude two rapid single electron oxidations. Hydrolysis of *C*-nitrosoformamide (**2**) would yield a carbamic acid that decomposes to carbon dioxide, ammonia, and nitroxyl (HNO). Reductive nitrosylation of the ferric heme of catalase by HNO would form the ferrous NO−catalase complex. The autooxidation of the ferrous−NO catalase complex yields nitrate and ferric catalase (vide infra), which can be activated to Compound I and react with another hydroxyurea molecule.

Gas chromatographic measurements identify the production of carbon dioxide during this reaction but fail to show the formation of nitrous oxide, the dimerization and dehydration product of HNO and a common marker for the intermediacy of HNO.[37] These results are surprising as nitrous oxide has been identified during the similar oxidation of cyanamide by catalase and taken as evidence for HNO formation.[19] Control experiments show that nitrous oxide does not escape from the reaction vessel during incubation. The limit of detection of the nitrous oxide assay ensures the detection of less than 1% of the theoretical amount of nitrous oxide from this reaction under these conditions. We propose that the reductive nitrosylation of the ferric heme of catalase by HNO effectively competes with HNO dimerization and explains the lack of nitrous oxide formation. Ferric heme proteins act as effective traps of HNO that kinetically compete with HNO dimerization.[30,32] The formation of a nitroxyl−catalase complex has recently been proposed to explain the inability to detect HNO electrochemically (after oxidation to NO) during the oxidation of cyanamide with catalase.[38] The binding or reaction of cyanamide, but not hydroxyurea, with ferric catalase may effectively compete with the reductive nitrosylation by HNO allowing HNO dimerization and nitrous oxide formation, explaining the observed differences in nitrous oxide production in these reactions. The results of these studies clearly indicate the need for improved methods of free HNO detection.

Scheme 3 summarizes the reactions of the ferrous−NO catalase complex. Exposure of the ferrous−NO catalase complex to air results in the formation of nitrate and ferric heme as determined by chemiluminescence detection and absorption spectroscopy, respectively. The autooxidation of iron nitrosyl heme proteins to nitrate and the ferric heme is a general process and has previously been noted for the ferrous−NO catalase complex.[34,39] This process occurs at a rate that allows for reactivation of the enzyme during the reaction with hydroxyurea and explains the observed mixture of ferric catalase and ferrous−NO catalase and the preponderance of nitrate. The possible oxidation of nitrite by catalase to nitrate could also explain the high nitrate-to-nitrite ratio.[35,36]

Absorption studies show the conversion of oxyHb to metHb during the catalase-mediated oxidation of hydroxyurea. While hydroxyurea also converts oxyHb to metHb, the slow rate of this reaction does not account for the observed conversion of oxyHb to metHb during the reaction of catalase and hydroxyurea in the presence of a peroxide generating system.[26] These results indicate that the ferrous−NO complex of catalase formed during the reaction with hydroxyurea releases NO that reacts with oxyHb and suggests a weak association of NO to the ferrous heme iron. The catalase−NO complex derived from the reaction of catalase and azide also releases NO that reacts with oxyHb.[38] To further illustrate the ability of the ferrous−NO catalase complex to release NO, EPR experiments show that the ferrous−NO catalase complex, prepared from ferric catalase and Angeli's salt, converts the nitric oxide specific EPR trap, carboxy-PTIO to carboxy-PTI. Overall, these results reveal that the combination of ferric catalase and HNO, derived from either hydroxyurea or Angeli's salt, represents a NO donor through the intermediacy of a ferrous−NO catalase complex.

The formation of a ferrous−NO catalase complex capable of NO donation from the reaction of hydroxyurea and catalase could have important implications in hydroxyurea based sickle cell disease therapy. Both animal models and patients receiving hydroxyurea demonstrate an increase in NO metabolites, indicating the in vivo ability of hydroxyurea to act as an NO donor.[8,9,40] Catalase-mediated release of NO from hydroxyurea could benefit patients through various mechanisms such as decreasing platelet adhesion or scavenging cell-free hemoglobin.[7,41] In addition, recent experiments indicate that hydroxyurea and other NO donors increase fetal hemoglobin production at least in part through a soluble guanylate cyclase-dependent pathway.[11] Complexes of NO and catalase, such as those formed in the described reaction of hydroxyurea and catalase, activate soluble guanylate cyclase in a manner similar to free NO and could ultimately stimulate fetal hemoglobin synthesis.[16,17] While these results clearly show the ability of catalase, an enzyme found in high levels in both liver and erythrocytes, to convert hydroxyurea to NO, further studies will be required to identify the role catalase plays during in vivo metabolism of hydroxyurea.

Summary

Hydroxyurea is currently used as a therapy for sickle cell disease, and recent evidence indicates that a portion of its beneficial effects may be related to hydroxyurea's ability to release nitric oxide (NO). While patients

taking hydroxyurea show measurable increases in a number of NO-derived metabolites,[8,9] the site and mechanism for the conversion of hydroxyurea to NO remains poorly undefined. These results identify the enzyme catalase as a potential candidate for the in vivo oxidation of hydroxyurea to NO. Spectroscopic and product analysis studies strongly suggest the initial conversion of hydroxyurea to nitroxyl (HNO) that reductively nitrosylates the ferric heme of catalase to form a ferrous−NO catalase complex. This complex releases NO that reacts with oxyHb and an NO-specific EPR trap. The formation of such ferrous−NO complexes could play a role in the beneficial effects of hydroxyurea in sickle cell disease.

Experimental Section

Materials. Hydroxyurea, 30% hydrogen peroxide, 3-amino-1,2,4-triazole, catalase (Sigma C-40, bovine liver), and glucose oxidase were purchased from Sigma Chemical Company, St. Louis, MO. 1,3-Cyclohexadiene and D-glucose were purchased from Aldrich Chemical Co., Milwaukee, WI. 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 ($Na_2N_2O_3$, Angeli's Salt) were purchased from Cayman Chemical Company, Ann Arbor, MI. Normal adult hemoglobin was isolated and purified as previously described.[13]

Nitrite/Nitrate Measurements. Catalase (1.0 μM) was added to a solution of hydroxyurea (1 mM), glucose (10 mM), and glucose oxidase (7.5 units) in phosphate buffer (100 mM, pH 7.4, 0.1 mL). After incubation at 37 °C for 3 h, an aliquot (5 μL) of the reaction mixture was injected into the reaction vessel of a Sievers 280 Nitric Oxide Analyzer chemiluminescence detector, and nitrite and nitrate were measured as reported earlier.[13] Identical experiments were performed using heat-inactivated (95 °C, 30 min) catalase or in the presence of 3-amino-1,2,4-triazole (200 mM). Control experiments were performed in the absence of either glucose/glucose oxidase or catalase.

Spectroscopy of the Reaction of Hydroxyurea and Catalase. Catalase (2 μM) was added to a solution of hydroxyurea (50 mM), glucose (10 mM), and glucose oxidase (15 units) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature. UV−vis measurements were taken every 5 min on a Cary 100 Bio UV−visible Spectrophotometer (Varian, Walnut Creek, CA). Similarly, hydroxyurea (50 mM) was added to a solution of catalase (0. 8 mM), glucose (10 mM), and glucose oxidase (7.5 units) in phosphate buffer (100 mM, pH 7.4, 0.3 mL) at room temperature. After 3 h, this solution was transferred to an EPR tube and frozen in liquid nitrogen. EPR spectra were taken at 130 K on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.37 GHz microwave frequency with *g*-values being determined from a superimposed spectrum of 2,2-diphenyl-1-picrylhydrazyl, $g = 2.0036$.

Gas Chromatographic Analysis of the Reaction Headspace. Catalase (10 μM) was added to a solution of hydroxyurea (500 mM), glucose (100 mM), and glucose oxidase (15 units) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature in a 20 mL flask with a rubber septum. After 3 h, an aliquot of the reaction headspace (250 μL) was injected onto a 6890 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector and 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 time of carbon dioxide was 2.10 min and identical to a known sample. Using a standard mixture of 10% N_2O in helium (Matheson), the limit of detection of this assay is 9 nmol of N_2O. Control experiments show no loss of N_2O over 3 h from the reaction vessel

Cycloadduct (3) Trapping. 1,3-Cyclohexadiene (19.0 μL, 0.1 mmol, 50 mM) was added to a solution of hydroxyurea (500 mM), catalase (10 μM), glucose (20 mM), and glucose oxidase (15 units) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature. The reaction was monitored by TLC, and after 3 h the reaction mixture was extracted with EtOAc (2 × 2.0 mL), dried over $MgSO_4$, and evaporated to give cycloadduct (**3**) as a residue: TLC R_f 0.26 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$, TMS internal standard) δ 6.4 (m, 2H), δ 4.7 (s, 1H), δ 4.9 (s, 1H), δ 1.4 (m, 4H).

Reaction of Sodium Trioxodinitrate with Catalase. A degassed solution of sodium trioxodinitrate (Angeli's salt, 4 μL, 10 mM) in 0.01 M NaOH was added to a degassed solution of catalase (10 μM) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) at room temperature in a cuvette with a rubber septum and UV−vis measurements were taken every 5 min. UV−vis and chemiluminescence nitrite/nitrate measurements were made on this solution upon exposure to air. Similarly, a degassed solution of sodium trioxodinitrate (8 μL, 0.1 M) in 0.01 M NaOH was added to a degassed solution of catalase (0.8 mM) in phosphate buffer (100 mM, pH 7.4, 1.0 mL) at room temperature and transferred to an EPR tube and frozen in liquid nitrogen and EPR spectra were taken at 130 K. In addition, NO saturated buffer (100 μL, ∼2 mM) was added to a degassed solution of catalase (0.8 mM) in phosphate buffer (100 mM, pH 7.4, 0.4 mL) at room temperature and transferred to an EPR tube and frozen in liquid nitrogen.

NO Release from Catalase (Fe^{2+})−NO and Reaction with Oxyhemoglobin. Catalase (1 μM) was added to a solution of hydroxyurea (50 mM), glucose (100 μM), glucose oxidase (2 units), and oxyhemoglobin (20 μM) in phosphate buffer (100 mM, pH 7.4, 2.0 mL) in a cuvette at room temperature. The conversion of oxy to methemoglobin was monitored using UV spectroscopy with measurements being taken every 5 min.

EPR Detection of NO Release from Catalase (Fe^{2+})−NO Complex. A solution of Angeli's salt (2 μL, 0.1M) in 0.01 M NaOH was added to a degassed solution of catalase (5 μM) in phosphate buffer (100 mM, pH 7.4, 0.2 mL) at room temperature. After 10 min, a solution of carboxy-PTIO (20 μL, 1 mM) was added, and the mixture was transferred to a capillary tube. EPR spectra were taken at room temperature using 8.5 mW microwave power, 1.25 G modulation amplitude, and 9.42 GHz microwave frequency.

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Supporting Information Available: Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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