Hydroxyurea Analogues As Kinetic and Mechanistic Probes of the Nitric Oxide Producing Reactions of Hydroxyurea and Oxyhemoglobin

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Derivatives of *N*-hydroxyurea that contain an N-hydroxy group react with oxyhemoglobin to form methemoglobin and variable amounts of nitrite/nitrate. Compounds with an unsubstituted -NHOH group produce the most nitrite/nitrate, which provides evidence for nitric oxide formation. The rate of reaction of these *N*-hydroxyurea derivatives with oxyhemoglobin correlates well with that compound's oxidation potential. Aromatic *N*-hydroxyureas react 25−80-fold faster with oxyhemoglobin than with *N*-hydroxyurea, suggesting other *N*-hydroxyurea analogues may be superior nitric oxide donors. Electron paramagnetic resonance spectroscopy shows that the formation of a low-spin methemoglobin−hydroxyurea complex is critical for iron nitrosyl hemoglobin formation. These results show that iron nitrosyl hemoglobin formation from the reaction of hydroxyureas and hemoglobin requires an unsubstituted -NHOH group and that the nitrogen atom of the non-N-hydroxy group must contain at least a single hydrogen atom. These results should guide the development of new hydroxyurea-based nitric oxide donors and sickle cell disease therapies.

Introduction

Hydroxyurea (**1**) is a recently approved drug for treating sickle cell disease,[1,2] a condition that affects about one out of six hundred people of African descent born in the United States.[3] Hydroxyurea benefits sickle cell patients by increasing the level of fetal hemoglobin (HbF) a genetically distinct hemoglobin that inhibits polymerization of deoxygenated sickle cell hemoglobin (HbS).[1,2,4] In addition, hydroxyurea may benefit sickle cell patients by influencing red cell−endothelial cell interactions, the rheological properties of red cells, and through myelosuppressive actions.[4] Hydroxyurea also acts as a source of nitric oxide (NO), a biologically important messenger molecule involved in the maintenance of normal blood pressure and flow that has drawn considerable interest as a sickle cell disease therapy.[5−7]

Electron paramagnetic resonance (EPR) studies demonstrate the in vivo formation of iron nitrosyl hemoglobin (HbNO) in the blood of both rats and humans upon the administration of hydroxyurea.[8,9] Sickle cell patients on hydroxyurea therapy show increased plasma and red cell nitrite, nitrate, HbNO, and cyclic guanylate monophosphate (cGMP) levels.[10,11] These results clearly associate hydroxyurea therapy with NO generation, which could potentially benefit sickle cell patients in a number of ways.[6,7] Recent in vitro work reveals that hydroxyurea and two other mechanistically different NO donors increase γ-globin gene expression and HbF levels in human erythroid progenitor cells by a soluble guanylate cyclase (sGC) dependent pathway providing further evidence for a role of NO in hydroxyurea therapy.[12]

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The in vitro reaction of hydroxyurea with normal oxyhemoglobin (oxyHb) produces methemoglobin (metHb) as the major protein product and HbNO as a minor product along with nitrate and nitrite.[13] Cyanide trapping studies indicate that the formation of HbNO from this reaction proceeds through the intermediacy of metHb.[13] EPR studies show the formation of the nitroxide radical (**2**, Scheme 1), which has been proposed to decompose to NO,[14] during the reaction of hydroxyurea and oxyHb.[15] Direct incubation of hydroxyurea with metHb initially produces an EPR detectable low spin hydroxyurea−metHb complex that ultimately forms HbNO.[13] Scheme 1 depicts a general model for the reactions of hydroxyurea and oxy and metHb with subsequent HbNO formation.[13] For oxyHb, hydrogen atom abstraction from hydroxyurea by the oxygen bound to the iron of oxyHb produces metHb, hydrogen peroxide, and the nitroxide radical (**2**, first reaction, Scheme 1). Hydroxyurea coordinates to the ferric iron of metHb and inner sphere electron transfer produces deoxyHb and the nitroxide radical (**2**) and deoxyHb (second reaction, Scheme 1). Nitric oxide formed from the decomposition of **2** rapidly combines with deoxyHb to give HbNO or oxidizes remaining oxyHb to metHb and nitrate (third reaction, Scheme 1). These results and model find precedence in the known conversion of oxyHb to deoxyHb through the intermediacy of metHb by many organic reductants including phenylhydroxylamine.[16]

Scheme 1 indicates that ease of oxidation of hydroxyurea and its ability to bind the ferric iron of metHb should play important roles in the formation of NO and HbNO from its reaction with oxyHb. Using a combination of electrochemical, kinetic, and spectroscopic measurements and a group of synthetic hydroxyurea derivatives (**3**−**8**) (Chart 1), we have investigated how structure influences these factors in relationship to a compound's ability to produce NO and HbNO. These

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Scheme 1

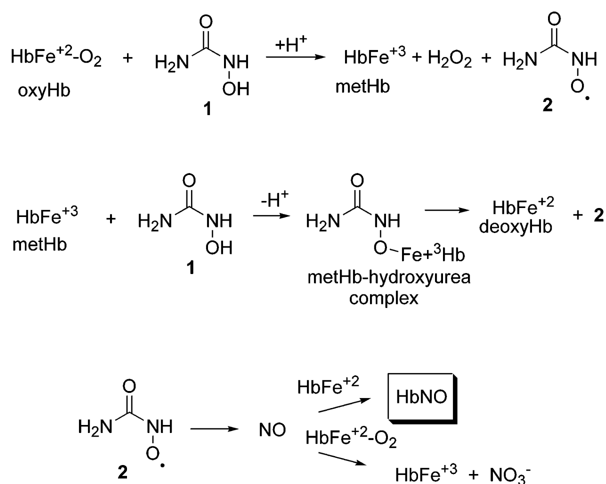
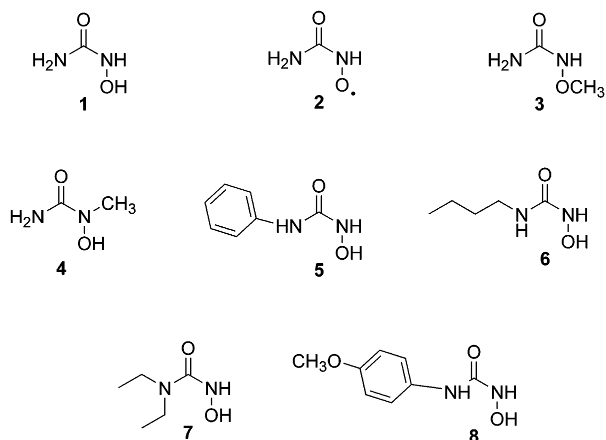


Chart 1



studies show that the structure of the hydroxyurea derivative directly controls both the ability of the compound to generate NO and HbNO and the rate of NO release. Such information may ultimately be useful for the design and preparation of hydroxyurea derivatives with improved NO releasing profiles.

Results

Absorption measurements indicate the formation of mainly metHb from the reaction of normal oxyHb with an excess of **4**–**8** (Figure 1). Figure 1 shows a representative scanning kinetic spectra from the reaction of oxyHb and **5** where the increasing absorbance at 500 and 635 nm and the decreasing absorbance at 540 and 577 nm indicate the conversion of oxyHb to metHb. The reactions of oxyHb and **5** and **6** occur appreciably faster than the reaction of oxyHb and **1** and demonstrate increased levels of turbidity and protein precipitation. In general, these results parallel previous results from absorption studies that show that **1** reacts with oxyHb to predominately form metHb.[13] Analogue **3** does not react with oxyHb and **4** converts oxyHb to metHb as previously demonstrated.[13]

The various hydroxyurea derivatives react with oxyHb at different rates and singular value decomposition (SVD) and global analysis of the change in absorption over time provide apparent rate constants for the reactions. Table 1 summarizes the apparent rate constants for the reactions between oxyHb and the various

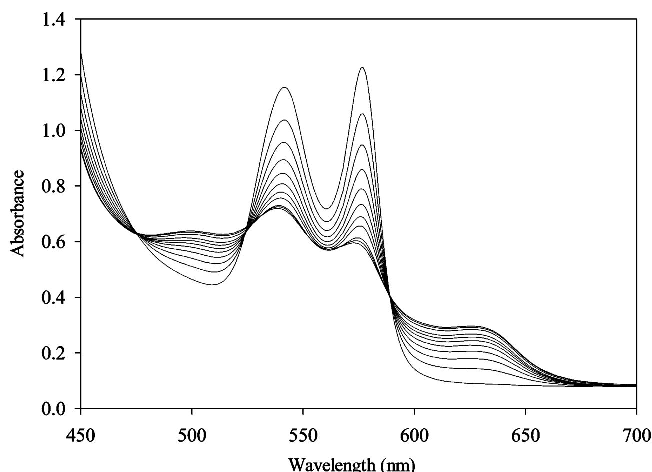


Figure 1. Absorption scanning kinetics of the reaction of oxyHbA (70 μM) and 50 mM *N*-phenyl *N*-hydroxyurea (**5**) in 0.1 M sodium phosphate buffer (pH 7.3) at room temperature (25 °C) under an atmosphere of air. Scans taken every 5 min total for 3 h.

Table 1. Apparent Rate Constants for the Reactions of Hydroxyurea Derivatives (50 mM) with OxyHb ($x \pm$ SD, $n = 3$), Chemical Potentials and p*K*a's of Hydroxyurea (**1**) and Derivatives (**4**–**8**)

derivative	apparent rate constant (min^{-1})	$E_{p2/2}$ (mV)[a]	pK_a
1	$7.54 \times 10^{-4} \pm 2.16 \times 10^{-5}$	948.0	10.65
4	$3.93 \times 10^{-2} \pm 1.68 \times 10^{-3}$	588.0	9.95
5	$6.24 \times 10^{-2} \pm 6.18 \times 10^{-3}$	433.0	10.30
6	$8.56 \times 10^{-3} \pm 1.87 \times 10^{-4}$	693.0	11.00
7	$2.26 \times 10^{-3} \pm 8.16 \times 10^{-5}$	705.0	10.80
8	$1.94 \times 10^{-3} \pm 5.50 \times 10^{-4}$[b]	560.0	10.20

[a] Half-peak potential vs Ag/AgCl. [b] 5 mM.

hydroxyurea derivatives (50 mM). Such analysis gives a rate constant for the reaction of **1** with oxyHb similar to that previously reported.[17,18] Similar to **1**, the rate constants of reactions of oxyHbS and the various hydroxyurea derivatives remain similar to the rate constants of the corresponding reaction with oxyHb (data not shown).[17,18] Due to poor solubility, the reaction of *N*-4-methoxyphenyl *N*-hydroxyurea (**8**) with oxyHb was performed at a lower concentration (5 mM).

Table 1 summarizes the chemical potential (Ep/2, half peak potential) of each of the hydroxyurea derivatives (**1**, **4**–**8**) as determined by cyclic voltammetry (CV). Consistent with an earlier report, hydroxyurea and its derivatives (**4**–**8**) demonstrate irreversible redox behavior and the measured value for hydroxyurea compares well to a previously reported value.[19] Table 1 shows that the compounds with the lowest chemical potentials (easiest to oxidize) react faster with oxyHb than compounds with higher chemical potentials. Hydroxyurea (**1**) reacts the slowest and is the most difficult to oxidize of all of the hydroxyureas examined, while the aromatic hydroxyureas (**5** and **8**) oxidize the easiest and react the fastest (adjusted for the concentration of **8**). Titration measurements provide the pK_a's of each of the hydroxyurea derivatives (**1**, **4**–**8**, Table 1). These experiments show that the first pK_a for hydroxyurea is 10.65, which compares well with a previously reported pK_a of 10.60 for hydroxyurea.[20,21] The first pK_a's of hydroxyurea and its derivatives (**4**–**8**) all fall between 9.95 and 11.00, indicating that these compounds pre-

Table 2. Nitrite and Nitrate Production from the Reactions of OxyHb with Hydroxyurea Derivatives (50 mM, $n = 3$)

derivative	NO_2^- (μM)	NO_3^- (μM)	HbNO	MetHb complex
1	9.4 ± 0.6	25.1 ± 0.5	yes	yes
3	0	0	no	no
4	6.3 ± 0.5	7.5 ± 1.1	no	yes
5	7.3 ± 1.1	26.1 ± 2.3	yes	yes
6	2.4 ± 0.8	3.5 ± 1.3	yes	yes
7	6.1 ± 0.5	18.5 ± 1.2	no	no

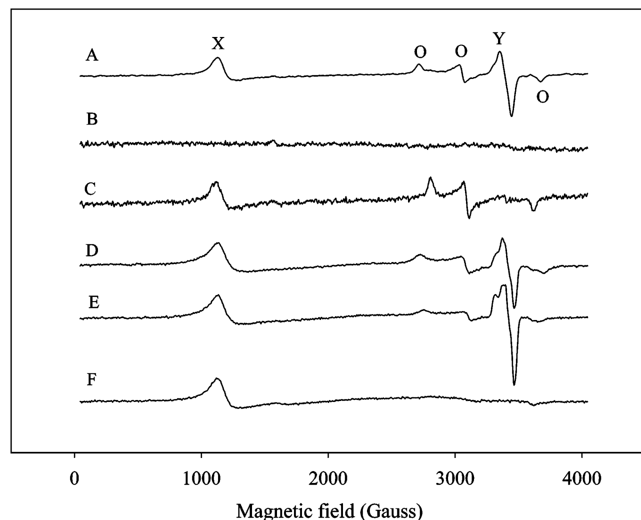


Figure 2. EPR spectra for the reactions of hydroxyurea derivatives with oxyhemoglobin. Hemoglobin solutions (1−2 mM in heme) were treated with hydroxyurea derivatives (50 mM) in 0.1 M sodium phosphate buffer (pH 7.3) and allowed to react for 12 h at room temperature. These reaction solutions were transferred to an EPR tube and frozen in liquid nitrogen (77 K). EPR spectra were taken on a Bruker ER 200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency. (A) The reaction of oxyHbA and **1**. (B) The reaction of oxyHbA and **3**. (C) The reaction of oxyHbA and **4**. (D) The reaction of oxyHbA and **5**. (E) The reaction of oxyHbA and **6**. (F) The reaction of oxyHbA and **7**. For all spectra: X = high spin metHbA, O = low spin metHbA hydroxyurea complex, and Y = iron nitrosyl hemoglobin (HbNO).

dominately exist in the un-ionized form at pH 7.3 (used for both kinetic and CV measurements).

Chemiluminescence NO analysis following reaction mixture reduction with a potassium iodide/glacial acetic acid solution or a refluxing vanadium (III) chloride/HCl solution provides a method for the measurement of the stable oxidation decomposition products of NO, nitrite, and nitrate (through subtraction). Table 2 summarizes the production of nitrite and nitrate from the reactions of the derivatives (**3**−**7**) with oxyHb in comparison with hydroxyurea. The reactions of oxyHb with all the derivatives except **3** produce both nitrite and nitrate, and no large differences exist in reactions in which HbS is used in place of Hb (data not shown). Except for derivatives **4** and **6**, the reactions with oxyHb produce much more nitrate than nitrite, and the amount of nitrate formed increases with hydroxyurea concentration.[13]

EPR experiments reveal the formation of HbNO and metHb from the reaction of hydroxyurea and its derivatives with oxyHb (1−2 mM). Figure 2 shows the EPR spectra from the frozen (77 K) reaction mixtures of oxyHb and the hydroxyurea derivatives. The reaction

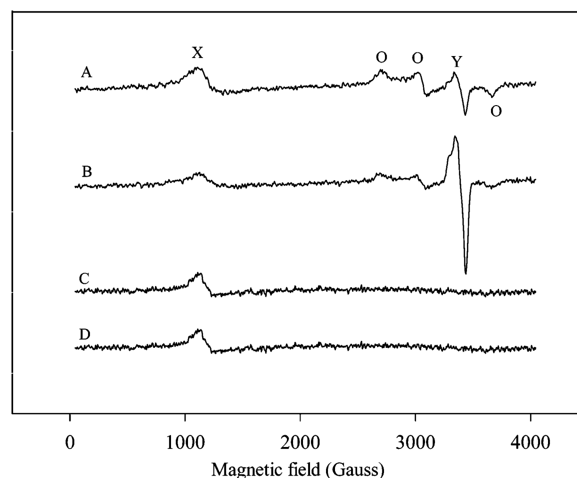


Figure 3. EPR spectra for the reactions of hydroxyurea derivatives with methemoglobin. Methemoglobin solutions (1−2 mM in heme) were treated with hydroxyurea derivatives (50 mM) in 0.1 M sodium phosphate buffer (pH 7.3) and allowed to react for 5 min or 12 h at room temperature under air. (A) EPR spectrum of the reaction of metHbA and **5** after 5 min, (B) EPR spectrum of the reaction of metHbA and **5** after 12 h, (C) EPR spectrum of the reaction of metHbA and **7** after 5 min, (D) EPR spectrum of the reaction of metHbA and **7** after 12 h. EPR spectra were taken on a Bruker ER 200D spectrometer using identical conditions described in Figure 2. For all spectra: X = high spin metHbA, O = low spin metHbA hydroxyurea derivative complex, and Y = HbNO.

of hydroxyurea and oxyHb produces an EPR spectrum characterized by resonances at $g = 2.01$, corresponding to HbNO, $g = 6$ for high-spin metHb and $g = 2.52$, 2.24, and 1.86 for a low-spin metHb−hydroxyurea complex (Figure 2A).[13] Consistent with the absorbance experiments, **3** does not react with oxyHb (Figure 2B).[13] The reaction of *N*-methyl *N*-hydroxyurea (**4**) with oxyHb only produces metHb species and no HbNO (Figure 2C).[13] The reactions of oxyHb with derivatives **5** and **6** produce spectra nearly identical to the reaction of hydroxyurea, which indicate the formation of HbNO, high-spin metHb, and a low-spin metHb−hydroxyurea complex (Figure 2D,E). *N*′,*N*′-diethyl *N*-hydroxyurea (**7**) only produces high-spin metHbA; no low-spin metHbA−hydroxyurea complex or HbNO form during this reaction (Figure 2F). The poor water solubility of **8** limited our ability to perform EPR measurements, which require higher concentrations of both protein and hydroxyurea analogue. Table 2 also summarizes HbNO and metHb formation from the reactions of these compounds with oxyHb.

Further EPR measurements show the ability of the hydroxyurea derivatives to form a low-spin complex with metHb. Previous work shows the rapid formation of a low-spin metHb−hydroxyurea complex upon addition of either hydroxyurea or derivative **4** to metHb.[13] The hydroxyurea and metHb mixture produces HbNO over time, while the reaction with **4** fails to form any HbNO.[13] Similar to hydroxyurea, the addition of derivatives **5** and **6** to metHb rapidly produces a low-spin metHb−hydroxyurea complex (within 5 min) and HbNO over 12 h (Figure 3A,B). The reaction of **5** and metHb actually produces some HbNO within 5 min (Figure 3A). The addition of *N*′,*N*′-diethyl *N*-hydroxyurea (**7**) to metHb does not form any low spin-metHb complex or HbNO even after 12 h (Figure 3C,D).

Discussion

These results provide further support for Scheme 1 as a mechanistic pathway of NO and HbNO formation from the reaction of hydroxyurea and oxyHb and identify structural features important for both NO release and HbNO formation. As the hydroxyurea derivatives (**4**–**8**) each contain an N-hydroxy group, Scheme 1 predicts that oxyHb will oxidize these compounds in a similar fashion as hydroxyurea and other organic reductants.[13,16] Absorption spectroscopy experiments show that oxyHb oxidizes each of these derivatives with the formation of metHb. The inability of *O*-methyl hydroxyurea (**3**) to react with oxyHb further supports Scheme 1.

Absorption spectroscopy provides kinetic information regarding these reactions, and cyclic voltammetry reveals a direct relationship between the rate of these reactions and the ease of oxidation of the hydroxyurea derivatives. Compounds with the lowest oxidation potentials (most easily oxidized) react the fastest with oxyHb (Table 1). These results also support hydrogen atom abstraction as the first step in the reaction between hydroxyurea and oxyHb (Scheme 1, first reaction). The structure of the hydroxyurea influences the rate of reaction with oxyHb, and in general the addition of any alkyl or aryl group to either nitrogen atom of hydroxyurea increases the rate (Table 1). Specifically, the greatest rate increases occur with the addition of a methyl group to the N-hydroxy nitrogen (**4**) or the addition of aromatic groups to the other (non-N-hydroxy nitrogen, **5** and **8**, adjusted for the difference in concentration, Table 1). The rate increases observed with these derivatives most likely reflect the ability of these groups to stabilize the initially formed nitroxide radical (analogous to **2**) from the hydrogen atom abstraction of the parent hydroxyurea (Scheme 1, reaction 1). These results demonstrate the potential of these substituted hydroxyureas as superior NO donors compared to the parent (**1**).

The reactions of hydroxyurea and the derivatives (**4**–**7**) with oxyHb also produce nitrite and nitrate providing evidence for NO release (Table 2). The formation of NO from the proposed decomposition of nitroxide radicals, such as **2**, provides a mechanism for the formation of these species.[14] Recent support for NO production from the direct decomposition of **2** comes from work that shows the formation of NO and formamide from the Cu(II) oxidation of hydroxyurea.[22] Reaction of hydroxyurea-derived NO with deoxyHb would produce HbNO, with oxyHb would produce metHb and nitrate, and with oxygen would yield nitrite.[23,24] Gas chromatographic headspace measurements fail to identify the formation of nitrous oxide in these reactions arguing against the involvement of nitroxyl (HNO) in these reactions. The diminished amount of nitrite and nitrate formed in the reaction of **6** may be related to the significant protein precipitation observed in this reaction, which could limit nitrite/nitrate formation. The lack of nitrite/nitrate formation in the reaction of **3** with oxyHb further indicates the inability of this compound to undergo reaction.

While these results show the ability of hydroxyureas with an N-hydroxy group to produce NO during their reaction with oxyHb, EPR measurements indicate that only hydroxyureas (**1**, **5**, **6**) capable of forming a low-spin metHb–hydroxyurea complex ultimately produce HbNO (Figure 3, Table 2). These results suggest electron transfer within this complex reduces the iron and generates the corresponding nitroxide radical (**2** for hydroxyurea, Scheme 1, second reaction). Nitric oxide formation from this process within the heme pocket followed by rapid binding to the ferrous iron may eliminate competing reactions of NO with oxygen or oxyHb and explain HbNO formation only from hydroxyureas capable of forming a low-spin metHb complex (Scheme 1, reactions 2 and 3).[25] Direct addition of these hydroxyureas (**1**, **5**, **6**) to metHb also forms HbNO with the reactions of **5** and **6** with metHb producing HbNO within 5 min (Figure 3).[13] While these results clearly show the involvement of a low-spin metHb–hydroxyurea complex in the formation of HbNO; the precise details of electron transfer and NO release from nitroxide radicals, such as **2**, remain to be defined thus limiting mechanistic interpretations. In addition, the kinetics of HbNO formation during the reaction of these hydroxyureas and metHb remain to be fully explored.

EPR spectroscopy shows that **4** forms a low spin complex with metHb but that neither the reaction of **4** with oxyHb or metHb forms HbNO.[13] The failure to detect HbNO from these reactions indicates that **4** cannot efficiently transfer NO to the heme, and the lack of NO transfer in these reactions again most likely relates to the fact that formation of NO from **4** requires C–N bond cleavage that may not be kinetically compatible with HbNO formation.

Absorption spectroscopy shows that derivative **7** reacts with oxyHb to give metHb, but EPR experiments reveal that no HbNO forms during these reactions even though **7** contains an unsubstituted hydroxylamine group and produces nitrite/nitrate. A closer examination of the EPR spectrum for the reaction of **7** and oxyHb (Figure 2F) provides some understanding as to the lack of HbNO formation. This spectrum shows a resonance for high-spin metHb and no resonances for HbNO or a low-spin metHb complex. Figure 3 also shows that the mixture of **7** and metHb does not produce a metHb–hydroxyurea complex. As the formation of such a complex represents an important step in the proposed mechanism of HbNO formation from hydroxyurea, the failure of **7** to form such a complex and reduce the ferric iron would explain the lack of HbNO production in these reactions and support Scheme 1. Absorption spectroscopy of the reaction of metHb and **7** under an atmosphere of carbon monoxide fails to show the formation of any HbCO confirming the inability of **7** to reduce the iron atom.

The inability to form a metHb complex must be related to the structural differences between **1**, **4**–**7**. The compounds that form complexes with metHb (**1**, **4**–**6**) each have at least a single hydrogen atom on the non-hydroxy nitrogen of the hydroxyurea group. These compounds must be capable of arranging themselves in the heme pocket in a manner that allows iron coordination. However, in **7** both of the hydrogen atoms of this nitrogen are replaced with alkyl groups, and this added substitution apparently cannot be sterically tolerated and prevents the association of the compound with the iron. These results indicate that HbNO formation from

hydroxyureas require at least one hydrogen substituent on the non-hydroxy nitrogen of the urea group and further refine the structural requirements of HbNO formation.

The described reactivity of hydroxyurea and its analogues with oxyHb could have important implications in sickle cell disease therapy. Sickle cell patients demonstrate elevated levels of ferrous cell-free (outside of the red cell yet within the plasma) hemoglobin that rapidly scavenges NO thus reducing its bioavailability.[26] The ability of hydroxyurea to react with oxyHb to form metHb, which reacts slowly with NO,[27] and to produce NO that rapidly reacts with both oxy and deoxyHb to give metHb and HbNO could be an important mechanism of action of hydroxyurea in addition to its ability to stimulate HbF synthesis. The conversion of ferrous cell-free hemoglobin to cell-free metHb or cell-free HbNO through reactions with hydroxyurea or hydroxyurea-derived NO would allow endogenously produced NO to assume its normal function.

Experimental Section

Hydroxyurea was purchased from Aldrich Chemical CO (Milwaukee, WI). All other chemicals were of the highest analytical grade commercially available.

Preparation of Hemoglobin. HbS and normal adult hemoglobin (Hb) were prepared and stored at −80 °C as previously described.[13] Hemoglobin concentrations are expressed in terms of heme and were determined using previously reported extinction coefficients.[28]

Synthesis of Hydroxyurea Derivatives. *O*-Methyl *N*-hydroxyurea (**3**), *N*-methyl-*N*-hydroxyurea (**4**), *N'*-phenyl *N*-hydroxyurea (**5**), and *N'*-*n*-butyl *N*-hydroxyurea (**6**) were synthesized as previously described.[13,29,30]

Synthesis of *N*,*N*-Diethyl *N*-Hydroxyurea (7). *N*,*N*-diethylcarbamoyl chloride (3 g, 22.1 mmol, 2.8 mL) was added dropwise to a solution of hydroxylamine hydrochloride (1.8 g, 25.9 mmol) and potassium carbonate (2.5 g, 23.6 mmol) in ethyl acetate (15 mL) and distilled water (0.25 mL) at 0 °C with stirring. This solution was allowed to stand at room temperature overnight. After evaporation of the solvents, the crude product was purified by flash chromatography (ethyl acetate) to afford **7** as a white powder (1.7 g, 58%): mp 64−66 °C; ^{1}H NMR (300 MHz, CD_3SOCD_3) δ 8.76 (s, 1H), 7.87 (s, 1H), 3.15 (q, 4H, $J = 7$ Hz), 1.00 (t, 6H, $J = 7$ Hz); ^{13}C NMR (75 MHz, CD_3SOCD_3) δ 159.8, 40.3, 13.9. Anal. ($C_5H_{12}N_2O_2$) C, H, N.

Synthesis of *N'*-4-Methoxyphenyl *N*-Hydroxyurea (8). 4-Methoxyphenylisocyanate was substituted for phenylisocyanate in the previously described preparation of **5**:[29] mp 158−162 °C; ^{1}H NMR (300 MHz, CD_3SOCD_3) δ 8.86 (s, 1H), 8.74 (s, 1H), 8.61 (s, 1H), 7.54 (d, 2H, $J = 9$ Hz), 6.88 (d, 2H, $J = 9$ Hz), 3.75 (s, 3H); ^{13}C NMR (75 M Hz, CD_3SOCD_3) δ 163.3, 159.1, 136.8, 125.3, 118.1, 59.6. Anal. ($C_8H_{10}N_2O_3$) C, H, N.

Preparation of Hydroxyurea Derivatives Solutions. Solutions of hydroxyurea derivatives in sodium phosphate buffer (0.1 M, pH 7.3) were prepared fresh daily.

Absorption Spectroscopy. Hemoglobin solutions (70 μM, in heme) in 0.1 M sodium phosphate buffer (pH 7.3) were treated with hydroxyurea derivatives (50 mM) unless otherwise indicated. Absorption measurements were made every 5 min for 12 h or unless otherwise noted on a Cary 100 Bio UV−Visible spectrophotometer at 25 °C. Kinetic data was analyzed with Specfit (Spectrum Software Associates, Boston, MA) using singular value decomposition (SVD) and global analysis.[17,18] Rate constants for the reaction of each hydroxyurea derivative with hemoglobin were obtained in triplicate and averaged.

Electron Paramagnetic Spectroscopy. Hemoglobin solutions (1−2 mM in heme) were treated with hydroxyurea derivatives (50 mM) and allowed to react for 12 h at room temperature. These reaction solutions were transferred to an EPR tube and frozen in liquid nitrogen (77K) and EPR spectra were taken as previously described.[13]

Nitrite and Nitrate Analysis. Nitrite and nitrate were measured with a Sievers 280 Nitric Oxide Analyzer chemiluminescence detector as reported earlier.[13]

pK_a Determination of Hydroxyurea Derivatives. The pK_a's of the hydroxyurea derivatives were determined by titration as previously described.[20]

Electrochemistry of Hydroxyurea Derivatives. Cyclic voltammetry (CV) was carried out in a three-electrode minicell (2−3 mL) containing a glassy carbon-working electrode (diameter 1.5 mm), a platinum flag counter electrode, and a reference electrode (saturated Ag/AgCl) at a scan rate of 100 mV/s. Potentials were reported against the saturated Ag/AgCl electrode. Samples were typically 2.5 mM in 0.01 M phosphate buffer (pH 7.3) with 0.2 M $NaClO_4$ as the supporting electrolyte. Solutions were purged with argon prior to use and kept under argon during the experiment. The glassy carbon-working electrode was polished with 1.0, 0.3, and 0.05 μm α-alumina (Buehler Corp.) and rinsed with deionized water. This cleaning treatment was repeated using 0.05 μm α-alumina for polishing between runs. Data were collected using a Pine AFRDE4 (Pine Instrument Company, Grove City, PA) with bi-potentiostat/waveform generator.

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