Iron Nitrosyl Hemoglobin Formation from the Reactions of Hemoglobin and Hydroxyurea†

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ABSTRACT: Hydroxyurea represents an approved treatment for sickle cell anemia and acts as a nitric oxide donor under oxidative conditions in vitro. Electron paramagnetic resonance spectroscopy shows that hydroxyurea reacts with oxy-, deoxy-, and methemoglobin to produce 2−6% of iron nitrosyl hemoglobin. No *S*-nitrosohemoglobin forms during these reactions. Cyanide and carbon monoxide trapping studies reveal that hydroxyurea oxidizes deoxyhemoglobin to methemoglobin and reduces methemoglobin to deoxyhemoglobin. Similar experiments reveal that iron nitrosyl hemoglobin formation specifically occurs during the reaction of hydroxyurea and methemoglobin. Experiments with hydroxyurea analogues indicate that nitric oxide transfer requires an unsubstituted acylhydroxylamine group and that the reactions of hydroxyurea and deoxy- and methemoglobin likely proceed by inner-sphere mechanisms. The formation of nitrate during the reaction of hydroxyurea and oxyhemoglobin and the lack of nitrous oxide production in these reactions suggest the intermediacy of nitric oxide as opposed to its redox form nitroxyl. A mechanistic model that includes a redox cycle between deoxyhemoglobin and methemoglobin has been forwarded to explain these results that define the reactivity of hydroxyurea and hemoglobin. These direct nitric oxide producing reactions of hydroxyurea and hemoglobin may contribute to the overall pathophysiological properties of this drug.

Hydroxyurea (**1**) has emerged as an approved treatment for sickle cell anemia (*1*, *2*), a condition that affects about one out of six hundred people of African descent born in the United States (*3*). A portion of the beneficial effects of hydroxyurea treatment 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 (*1*, *2*). However, some 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 (*1*, *2*, *4*, *5*). Such results have focused efforts to define other mechanisms of action for hydroxyurea.

Recent reports indicate that hydroxyurea acts as a source of nitric oxide (NO),[1] a biologically important messenger molecule involved in the maintenance of normal blood pressure and flow. Chemical oxidation of hydroxyurea forms nitrite (NO_2^-) and nitrate (NO_3^-), the stable oxidative decomposition products of NO, and nitrous oxide (N_2O), which provides evidence for nitroxyl or the nitroxyl ion (HNO/NO^-) (*6*, *7*). Treatment of hydroxyurea with hydrogen peroxide and heme proteins produces a nitroxide radical (**2**), NO_2^-, and NO as judged by electron paramagnetic resonance (EPR) experiments using an NO-specific trap (*8*). Similar EPR studies show the production of NO during the oxidation of hydroxyurea with hydrogen peroxide in the presence of various copper-containing enzymes (*9*). The reaction of hydroxyurea and bovine oxyhemoglobin ($Fe^{2+}-O_2$) forms the nitroxide radical (**2**) and iron nitrosyl hemoglobin ($Fe^{2+}-NO$) (*10*). Recent EPR studies demonstrate the in vivo formation of HbNO in the blood of both rats and humans upon administration of hydroxyurea (*11*, *12*). 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) (*13*, *14*). As both NO and HbNO have been proposed as potential therapies for sickle cell disease (*15*−*17*), the formation of these products and SNO-Hb from the

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[1] Abbreviations: NO, nitric oxide; Hb, hemoglobin; HbA, normal adult hemoglobin; HbS, sickle cell hemoglobin; SNO-Hb, *S*-nitrosohemoglobin; HbNO, iron nitrosyl hemoglobin; HbCO, hemoglobin−carbon monoxide adduct; CNmetHb, cyanomethemoglobin; CO, carbon monoxide; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

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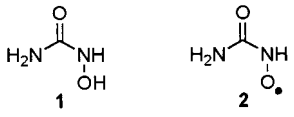
reaction of hydroxyurea and hemoglobin could explain a portion of hydroxyurea's effects.



We previously demonstrated the in vitro formation of sickle HbNO from the reaction of sickle cell oxyhemoglobin with hydroxyurea, and experiments using [^{15}N]hydroxyurea showed that the NO group attached to the protein directly derives from the drug (*7*, *18*). In addition, we showed that both sickle cell deoxyhemoglobin (Fe^{2+}) and methemoglobin (Fe^{3+}) reacted with hydroxyurea in vitro to form sickle HbNO and defined a ferric heme group as the minimum structural requirement for NO release from hydroxyurea (*19*, *20*). Such preliminary results reveal a complex reactivity of hydroxyurea with hemoglobin. Here we determine and quantify both the protein and hydroxyurea-derived products from the reactions of hydroxyurea and oxy-, deoxy-, and metHb in both the absence and presence of KCN and CO. In addition, similar experiments using hydroxyurea analogues provide information regarding the reaction mechanism and the structural requirements of NO release from hydroxyurea. Together, these results allow the development of a unified mechanistic model regarding NO and HbNO production from the reactions of hydroxyurea with hemoglobin.

MATERIALS AND METHODS

Hemoglobin Preparation. Excess, discarded blood was obtained that had been drawn from patients homozygous for the sickle gene with less than 5% fetal hemoglobin, following federal regulations and guidelines. The cells were washed several times in 0.95% NaCl and lysed by incubation in distilled water. The membranes were removed by centrifugation, and the supernatant was dialyzed against 0.01 M sodium phosphate buffer (pH 7.3). Normal adult hemoglobin was prepared similarly. The hemoglobin sample was pelleted in liquid nitrogen and stored at −80 °C. Hemoglobin samples used for experiments were thawed and used within 24 h. Thawed hemoglobin samples were further centrifuged to remove any precipitated or denatured protein. The absence of denatured protein was confirmed by absorption spectroscopy. Deoxyhemoglobin was formed by gently flowing argon over a solution of oxyHb in argon-saturated buffer in a cuvette with a rubber septum until the absorption spectrum showed the complete removal of oxygen. Human methemoglobin was purchased from Sigma Chemical Co. (St. Louis, MO) or prepared from oxyhemoglobin by treatment with excess potassium ferricyanide followed by gel filtration (Sephadex G-25). Hemoglobin concentrations are expressed in terms of heme and were determined using previously reported extinction coefficients (*21*).

Hydroxyurea Solutions. Hydroxyurea (Aldrich Chemical Co., Milwaukee, WI) solutions in 0.1 M sodium phosphate buffer (pH 7.3) were prepared fresh daily. Aged hydroxyurea solutions (>24 h old) produced similar end products as fresh hydroxyurea solutions but reacted faster. NMR analysis of an aged [^{15}N]hydroxyurea solution failed to indicate the presence of hydroxylamine, a hydrolysis product of hydroxyurea that reacts rapidly with hemoglobin (*22*−*24*). Anaerobic hydroxyurea solutions were prepared by bubbling argon for 20 min through the solution. Similar solutions of the hydroxyurea derivatives **3** and **4** in 0.1 M sodium phosphate buffer (pH 7.3) were also prepared fresh daily.

Nitric Oxide Saturated Buffer Solutions. Nitric oxide saturated buffer solutions were prepared by bubbling nitric oxide gas through a buffer solution (0.1 M sodium phosphate buffer, pH 7.3) in a small glass vial sealed with a rubber septa that had previously been deoxygenated by passing argon through it for 30 min. The nitric oxide was transferred from the tank to the vial using stainless steel lines and passed through a 1 M sodium hydroxide solution to remove any higher nitrogen oxides. Such solutions generally had a nitric oxide concentration of 1.7−2.0 mM as measured by chemiluminescence nitric oxide detection. Aliquots of this NO-saturated buffer were transferred by means of a gastight syringe.

Absorption Spectroscopy. Hemoglobin solutions (70 μM in heme) in 0.1 M sodium phosphate buffer (pH 7.3) were treated with hydroxyurea (0.5 M) and placed in a cuvette. Absorption measurements were made every 10 min for 12 h on a Cary 100 Bio UV−visible spectrophotometer at room temperature. For some experiments, KCN (0.5 M) was added to the reaction mixture. For nitric oxide control experiments, nitric oxide saturated buffer was added to hemoglobin solutions (1:20 v/v, final concentration of NO was about 100 μM) using a gastight syringe. A carbon monoxide atmosphere was achieved by gently blowing carbon monoxide over a reaction sample in a cuvette with a rubber septum in a fume hood.

Electron Paramagnetic Resonance Spectroscopy. Hemoglobin solutions (1−2 mM in heme) were treated with hydroxyurea (0.5 M) 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 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, $g = 2.0036$. The concentration of HbNO was determined on the basis of a standard curve generated by measuring the peak heights of known amounts of HbNO. For nitric oxide control experiments, nitric oxide saturated buffer was added to the hemoglobin solutions (1:1 v/v, final concentration of NO was about 1 mM) using a gastight syringe.

Nitrite, Nitrate, and S-Nitrosohemoglobin 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, nitrate, and *S*-nitrosothiol 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 on the basis of standard curves. For *S*-nitroso-

hemoglobin analysis, the reaction vessel contained a solution of L-cysteine and copper(I) chloride at 50 °C that converts *S*-nitrosothiols to nitric oxide (*25*).

Nitrous Oxide Analysis by Gas Chromatography. An aliquot of the headspace (250 μL) from a reaction performed in a glass vial sealed with a rubber septum 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 100 °C) with a flow rate of 16.67 mL/min (He, carrier gas). Under these conditions, a known sample of N_2O (Matheson) produced a peak with a retention time of 2.78 min, and the detection limit of this assay was 0.22 μmol.

^{13}C NMR Analysis of Urea. A solution of deoxyhemoglobin (75 μM) and argon-degassed hydroxyurea (0.5 M) in a glass reaction vessel sealed with a rubber septum and Parafilm was incubated at 37 °C. After 3 days, the reaction flask was heated to 90 °C by an oil bath until the protein completely denatured. Protein was removed by filtration and the remaining solution lyophilized to give a solid that was dissolved in deuterated DMSO and analyzed by ^{13}C NMR spectroscopy on a Bruker 300 Avance NMR spectrometer (ns = 6000, D1 = 2.0 s). The presence of urea was confirmed by the addition of urea (7.6 mg, 0.127 mmol) to the reaction sample followed by reanalysis.

*Synthesis of O-Methylhydroxyurea (**3**).* Potassium hydroxide (Aldrich Chemical Co., 507 mg, 9.04 mmol) was added to a solution of *O*-methylhydroxylamine hydrochloride (Aldrich Chemical Co., 724 mg, 8.67 mmol) in methanol (3 mL), and the resultant solution was stirred at room temperature for 45 min. The reaction solution was filtered into a cold stirred solution of trimethylsilyl isocyanate (Aldrich Chemical Co., 1.17 mL, 8.67 mmol) in THF (5 mL). The reaction mixture was stirred overnight at ambient temperature and then evaporated to dryness in vacuo. Diethyl ether (3 × 5 mL) was added and evaporated and the crude product dried in vacuo. The pale yellow product was twice recrystallized from hot methanol and ethyl acetate to yield colorless crystals of **3** (450 mg, 58%): mp 75−76 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.12 (s, 1H), 6.40 (s, 2H), 3.51 (s, 3H); ^{13}C NMR (75 MHz, DMSO) δ 161.17, 63.77. Anal. Calcd for $C_2H_6N_2O_2$: C, 26.66; H, 6.71; N, 31.10. Found: C, 26.25; H, 6.72; N, 30.64.

*Synthesis of N-Methylhydroxyurea (**4**).* Potassium hydroxide (507 mg, 9.04 mmol) was added to a solution of *N*-methylhydroxylamine hydrochloride (Aldrich Chemical Co., 724 mg, 8.67 mmol) in methanol (3 mL), and the resultant solution was stirred at room temperature for 45 min. The reaction solution was filtered into a cold stirred solution of trimethylsilyl isocyanate (1.17 mL, 8.67 mmol) in THF (5 mL). The reaction mixture was stirred overnight at ambient temperature and evaporated to dryness in vacuo. Diethyl ether (3 × 5 mL) was added and evaporated and the crude product dried in vacuo. The pale yellow product was twice recrystallized from hot methanol and ethyl acetate to yield colorless crystals of **4** (425 mg, 54.4%): mp 54−56 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.42 (s, 1H), 6.22 (s, 2H), 2.92 (s, 3H); ^{13}C NMR (75 MHz, DMSO) 162.46, δ 51.34. Anal. Calcd for $C_2H_6N_2O_2$ (90.0822): C, 26.66; H, 6.71; N, 31.10. Found: C, 26.38; H, 6.76; N, 30.81.

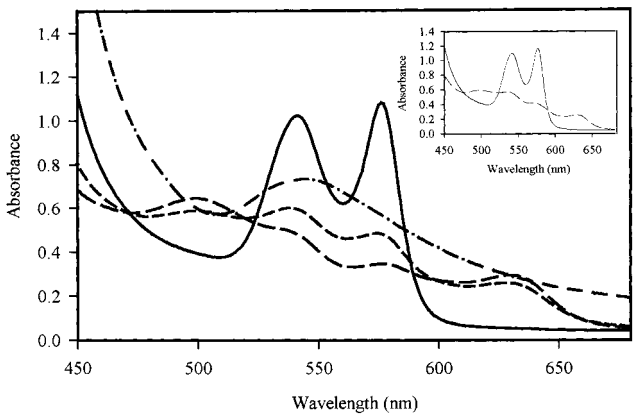


FIGURE 1: Absorption spectra of oxyHbA (solid line), metHbA (long dash line), the reaction of oxyHbA and hydroxyurea at room temperature under an atmosphere of air after 12 h (short dash line), and the reaction of oxyHbA and hydroxyurea in the presence of 0.5 M KCN (dash−dot line). The inset shows absorbance spectra of oxyHbA (solid line) and the reaction of oxyHbA and excess NO under an atmosphere of air.

RESULTS

Absorption measurements indicate the formation of mainly metHbA from the reaction of normal oxyHbA with an excess of hydroxyurea under an atmosphere of air (Figure 1). Increased absorbance at 500 and 635 nm and decreased absorbance at 540 and 577 nm demonstrate the conversion of oxyHbA to metHbA (Figure 1). Addition of NO to oxyHbA rapidly produces metHbA under these conditions (Figure 1, inset). EPR experiments reveal the formation of HbNO from the reaction of hydroxyurea and oxyHbA (1−2 mM, Figure 2A). A frozen reaction mixture (77 K) produces an EPR spectrum characterized by resonances at $g = 2.01$, corresponding to HbNO, $g = 6$ for high-spin metHbA, and $g = 2.52$, 2.24, and 1.86 for a low-spin metHbA−hydroxyurea complex (Figure 2A). This spectrum is identical to a reported EPR spectrum of a hydroxyurea and bovine oxyhemoglobin reaction mixture (*10*). Control EPR experiments of metHb in the same buffer (0.1 M sodium phosphate, pH 7.3) fail to show the resonances attributed to the low-spin metHbA−hydroxyurea complex, indicating that these signals do not arise from a pH-dependent metHb−hydroxyl ion complex (Figure 2C). EPR measurements of the reaction of NO with oxyHbA show the formation of only metHbA and no measurable HbNO under these conditions (Figure 2E). EPR measurements also indicate that 28 ± 2 μM HbNO (2−3% of the total hemoglobin) forms during this reaction. Experiments with oxyHbS produce identical absorption and EPR spectra and amounts of HbNO (21 ± 2 μM) similar to those from experiments with oxyHbA. Control EPR experiments also show that the original HbA and HbS samples do not contain HbNO and that HbNO does not form in the absence of hydroxyurea even after 48 h under the same experimental conditions.

Similar absorption measurements demonstrate the formation of mainly metHbA from the reaction of deoxyHbA with an excess of hydroxyurea (Figure 3). Increased absorbance at 500 and 635 nm and decreased absorbance at 555 nm indicate the conversion of deoxyHbA to metHbA. Addition of NO to deoxyHbA rapidly produces HbNO under these conditions (Figure 3, inset). EPR experiments again reveal the formation of 22 ± 2 μM HbNO (1.7% of the

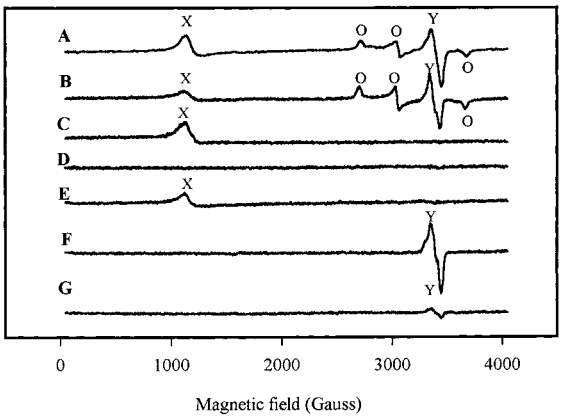


FIGURE 2: EPR spectra for the reactions of hydroxyurea or nitric oxide with hemoglobins. Hemoglobin solutions (1−2 mM in heme) were treated with hydroxyurea (500 mM) and allowed to react for 12 h at room temperature. Deoxyhemoglobin was formed by gently flowing argon over a solution of oxyHb as described in the Materials and Methods section and then treated with hydroxyurea for 12 h at room temperature under an argon atmosphere. For nitric oxide control experiments, NO saturated buffer (0.1 M sodium phosphate buffer, pH 7.3) was added to the hemoglobin solutions (1:1 v/v) and allowed to react under similar conditions. These reaction solutions were transferred to 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. (A) Reaction of oxyHbA and hydroxyurea under an air atmosphere. (B) Reaction of deoxyHb and hydroxyurea under an argon atmosphere. (C) MetHbA control spectrum. (D) Reaction of oxyHbA and hydroxyurea in the presence of 0.5 M KCN under an air atmosphere. (E) Reaction of oxyHbA and NO under an air atmosphere. (F) Reaction of deoxyHbA and NO under an argon atmosphere. (G) Reaction of metHbA and NO under an air atmosphere. For all spectra, X = high-spin metHbA, O = low-spin metHbA−hydroxyurea complex, and Y = iron nitrosyl hemoglobin (HbNO).

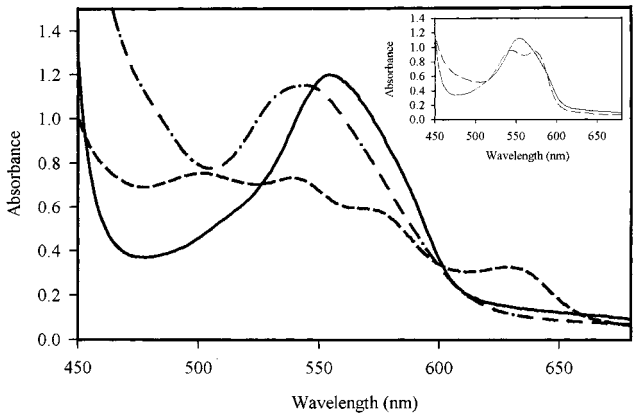


FIGURE 3: Absorbance spectra of deoxyHbA (solid line), the reaction of deoxyHbA with hydroxyurea under an argon atmosphere (short dash line), and the reaction of deoxyHbA with hydroxyurea in the presence of 0.5 M KCN under an argon atmosphere (dash−dot line). The inset shows absorbance spectra of deoxyHb (solid line) and the reaction of deoxyHb with excess NO under an argon atmosphere (short dash line).

total hemoglobin) from the reaction of hydroxyurea with deoxyHbA (1−2 mM). The EPR spectrum of the reaction of hydroxyurea and deoxyHbA is nearly identical to the spectrum of the reaction of hydroxyurea and oxyHbA (Figure 2B). Addition of NO to deoxyHbA forms only HbNO and no metHbA under these conditions (Figure 2F). Experiments with deoxyHbS produce results (29 ± 2 μM HbNO) similar to those from experiments with deoxyHbA.

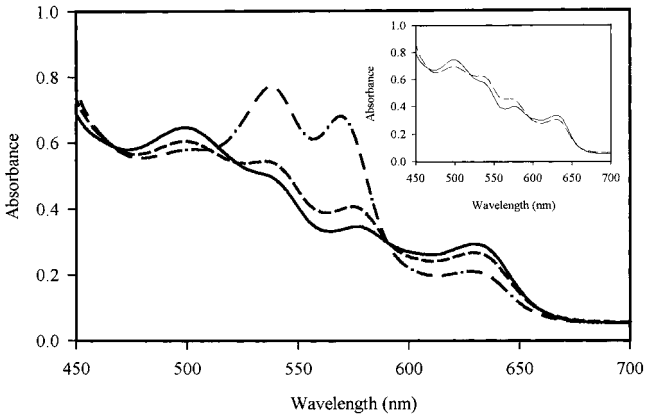


FIGURE 4: Absorption spectra of metHb (solid line), the reaction of metHb with hydroxyurea (short dash line) under an air atmosphere, and the reaction of metHb with hydroxyurea in the presence of CO (dash−dot line). The inset shows absorption spectra of metHb (solid line) and the reaction of metHb with NO under an air atmosphere (short dash line).

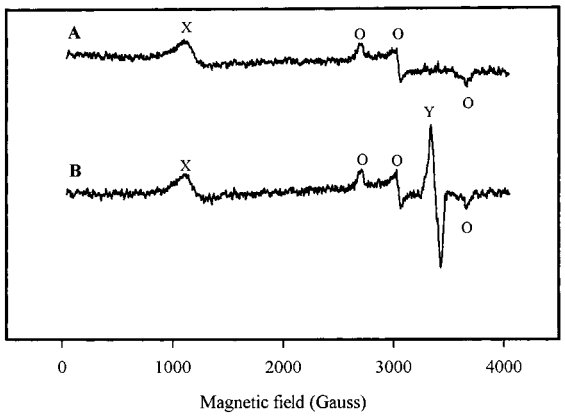


FIGURE 5: (A) EPR spectrum of the reaction of metHb and hydroxyurea after 5 min under an air atmosphere. (B) EPR spectrum of the reaction of metHb and hydroxyurea under an air atmosphere after 12 h. EPR spectra were taken on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency at 77 K. For all spectra, X = high-spin metHb, O = low-spin metHb−hydroxyurea complex, and Y = HbNO.

Addition of hydroxyurea to metHbA under an air atmosphere rapidly produces a small but evident change in the absorption spectrum as evidenced by the increasing absorbance at 537 and 575 nm (Figure 4). After these rapid changes, the absorption spectrum for the reaction of metHbA with hydroxyurea does not change significantly over time. Addition of NO to metHbA under an atmosphere of air also produces a final spectrum similar to that of metHbA (Figure 4, inset). However, EPR experiments clearly demonstrate the formation of 42 ± 3 μM HbNO (5.6% of the total hemoglobin, Figure 5B) from the reaction of hydroxyurea and metHbA under an air atmosphere. The EPR spectrum from this reaction is again similar to the EPR spectra from the reactions of hydroxyurea with both oxy- and deoxyHbA (Figures 2A,B and 5B). An EPR spectrum taken immediately after addition of hydroxyurea to metHbA only shows resonances for high-spin metHbA and the low-spin hydroxyurea−metHbA complex and did not show HbNO formation (Figure 5A). Addition of NO to metHbA under an air atmosphere produces a small amount of HbNO (Figure 2G).

Table 1: Nitrite and Nitrate Production from the Reactions of Hemoglobin with Hydroxyurea (**1**) and *N*-Methylhydroxyurea (**4**)

	NO_2^- (μM)	NO_3^- (μM)
oxyHbA + **1**	9.3 ± 1.8	88.2 ± 18.2
oxyHbS + **1**	13.7 ± 0.7	75.2 ± 12.6
deoxyHbA + **1**	4.6 ± 1.8	9.7 ± 1.9
deoxyHbS + **1**	10.2 ± 1.6	7.8 ± 1.6
metHbA + **1**	3.5 ± 0.5	7.4 ± 1.2
oxyHbA + **4**	6.3 ± 0.5	7.5 ± 1.1
metHbA + **4**	2.6 ± 0.4	2.2 ± 0.4

Absorption measurements of the reaction of metHbA with excess hydroxyurea under an atmosphere of CO show a mixture of the carbon monoxide adduct of hemoglobin (HbCO, Fe^{2+}), metHbA, and possibly small amounts of HbNO after 24 h (Figure 4). Increased absorbance at 540 and 569 nm indicates the presence of HbCO and possibly small amounts of HbNO, and the significant absorption at 500 and 635 nm demonstrates the presence of metHbA (Figure 4). Decomposition of the final spectra into its constituent species provides an estimate of the final composition of the reaction mixture. Fitting the final reaction spectra to the spectra of pure HbCO, HbNO, and the hydroxyurea−metHb complex indicates that the final mixture contains approximately 75% of the metHbA−hydroxyurea complex and 25% HbCO. Such analysis reveals only trace amounts of HbNO in this mixture. However, EPR measurements confirm the formation of HbNO (29 μM, 2.4%) during the reaction of hydroxyurea and metHbA under a CO atmosphere.

While the EPR spectra of the reactions of hydroxyurea with oxy-, deoxy-, and metHbA clearly show the formation of HbNO, the same reactions in the presence of KCN (0.5 M) produce only cyanomethemoglobin (CNmetHbA, Fe^{3+}) as judged by absorption spectroscopy (Figures 1 and 3). EPR experiments show that the presence of KCN completely suppresses HbNO formation during these reactions (Figure 2D).

Chemiluminescence NO detection following treatment of the oxyHb, deoxyHb, and metHb with hydroxyurea reaction mixtures with L-cysteine and copper(I) chloride at 50 °C provides a method for *S*-thiol detection (*25*). Such measurements do not reveal the presence of *S*-nitrosohemoglobin in these reactions.

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 measurement of the stable oxidative decomposition products of NO, nitrite, and nitrate, respectively. The reactions of oxyHbA or oxyHbS and hydroxyurea both produced amounts of nitrate similar to the concentration of the protein and much smaller amounts of nitrite (Table 1). The reaction of hydroxyurea and both oxy- and deoxyHbS forms significantly higher levels of nitrite than the corresponding reactions of hydroxyurea with oxy- and deoxyHbA. Under these conditions, nitrite and nitrate were not detected from control solutions of hemoglobin or hydroxyurea. Similar measurements from the reactions of deoxyHbA, deoxyHbS, and metHbA with hydroxyurea reveal much smaller amounts of nitrate and similar amounts of nitrite as compared to the reaction with oxyHbA (Table 1).

Gas chromatographic analysis of the reaction headspace allows for the detection of N_2O, the stable dimerization and dehydration product of nitroxyl (HNO). Such measurements fail to show the presence of N_2O in the reaction of hydroxyurea with oxyHbA, deoxyHbA, or metHbA.

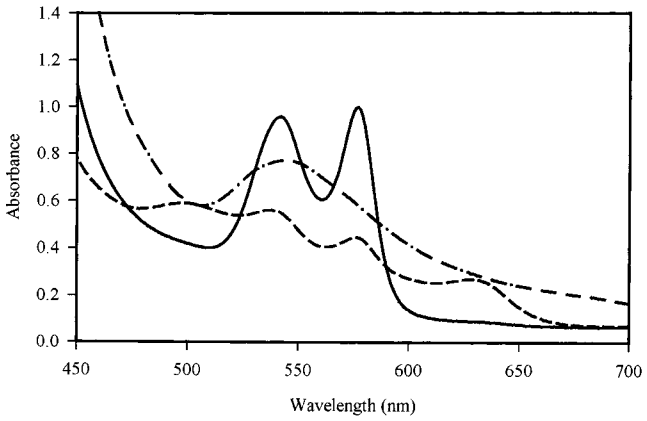
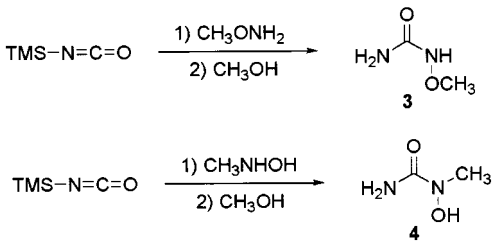


FIGURE 6: Absorption spectra of oxyHb (solid line), the reaction of oxyHb and **4** (short dash line), and the reaction of oxyHb and **4** in the presence of KCN (dash−dot line).

Scheme 1



Analysis of the reactions of hydroxyurea and oxyHbA or deoxyHbA by carbon-13 NMR spectroscopy shows a resonance at 160.1 ppm that indicates the formation of urea. Addition of a standard urea sample followed by reanalysis confirms the identity of urea in these reactions. Under identical conditions, urea was not found in control solutions of only oxyHbA, deoxyHbA, or hydroxyurea.

Chemical synthesis provides *O*-methylhydroxyurea (**3**) and *N*-methylhydroxyurea (**4**) as further mechanistic probes for the reaction of hydroxyurea and hemoglobin. Treatment of trimethylsilyl isocyanate with *O*-methylhydroxylamine or *N*-methylhydroxylamine followed by silyl group methanolysis produces **3** and **4** in 58% and 54% yield, respectively (Scheme 1).

O-Methylhydroxyurea (**3**, 50 mM) did not react with oxy-, deoxy-, or metHbA as judged by absorption spectroscopy. EPR experiments also show that no HbNO forms during the reaction of these hemoglobins with **3**. Chemiluminescence NO analysis indicates no nitrite or nitrate formation in these reactions.

N-Methylhydroxyurea (**4**, 50 mM) reacts with oxyHbA under an air atmosphere to form metHbA as judged by absorption spectroscopy (Figure 6). In the presence of KCN (0.5 M), this reaction only produces CNmetHbA (Figure 6). EPR analysis shows only high-spin metHbA and resonances similar to those of the metHbA−hydroxyurea complex and does not indicate any HbNO formation (Figure 7). Chemiluminescence analysis of this reaction mixture indicates the formation of small amounts of nitrite and nitrate (Table 1). The reaction of **4** with deoxyHbA also forms metHbA and does not produce HbNO as similarly determined by absorption and EPR spectroscopy.

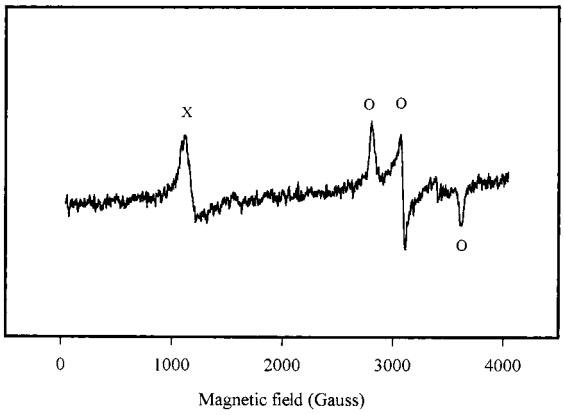


FIGURE 7: EPR spectrum of the reaction of oxyHb and **4**. X = high-spin metHb, and O = low-spin metHb−**4** complex. The EPR spectrum was taken on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency at 77 K.

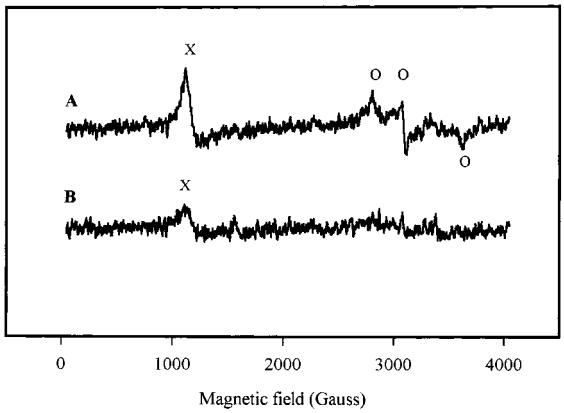


FIGURE 8: (A) EPR spectrum of the reaction of metHb and **4**. (B) EPR spectrum of the reaction of metHb and **4** in the presence of CO. EPR spectra were taken on a Bruker ER200D spectrometer using 8.5 mW microwave power, 5.0 G modulation amplitude, and 9.32 GHz microwave frequency at 77 K. For all spectra, X = high-spin metHb, and O = low-spin metHb−**4** complex.

The reaction of **4** with metHbA does not produce HbNO as determined by EPR spectroscopy (Figure 8A). The EPR spectrum from the reaction of **4** and metHbA only shows the presence of high-spin metHbA and resonances similar to those of the previously described metHbA−hydroxyurea complex (Figure 8A). Chemiluminescence analysis of this reaction mixture indicates the formation of small amounts of nitrite and nitrate (Table 1). Reaction of **4** and metHbA under a CO atmosphere clearly shows the formation of HbCO as indicated by the increased absorbance at 540 and 569 nm (Figure 9). EPR measurements of this reaction mixture show only a small amount of high-spin metHbA and no HbNO (Figure 8B).

DISCUSSION

Hydroxyurea reacts with oxyHbA, deoxyHbA, and metHbA in vitro and directly transfers NO to the iron atom to produce HbNO (2−6% of the total protein) as shown by EPR spectroscopy. The amount of HbNO formed in the reaction with oxyHb correlates well to our earlier estimates by absorption spectroscopy (*18*). Previous work shows that the NO attached to the heme iron derives from the NHOH group of hydroxyurea (*7*, *19*). Methemoglobin represents

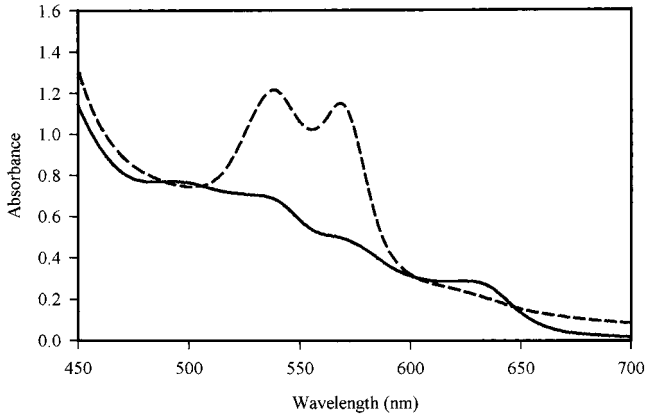


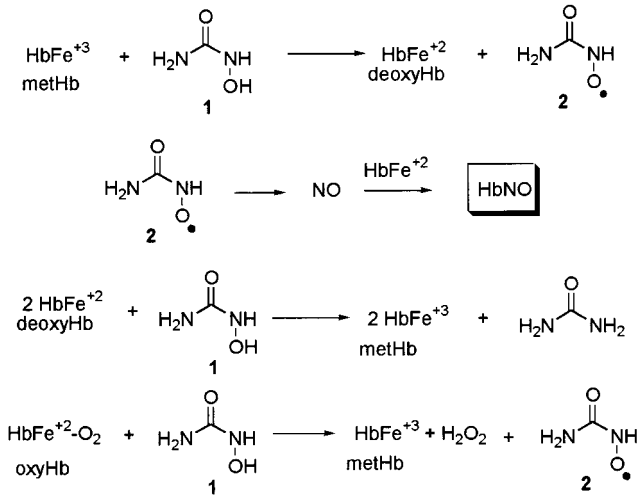
FIGURE 9: Absorption spectra of metHb (solid line) and the reaction of metHb and **4** in the presence of CO (dash line).

the major remaining protein product from these reactions. While these protein products could be envisioned to arise from the direct reactions of nitric oxide (NO) with oxyHbA, deoxyHbA, and metHbA, control experiments show that the direct addition of NO to these hemoglobins does not give results identical to those of the reactions with hydroxyurea. Under conditions identical to the reactions with hydroxyurea, EPR experiments show that the addition of NO to oxyHbA produces only metHbA and no detectable HbNO, the addition of NO to deoxyHbA forms only HbNO and no metHbA, and the addition of NO to metHbA produces some HbNO and no metHbA. Each of these results stands contrary to the observed products from the reactions with hydroxyurea. Also, control experiments indicate that hydroxyurea solutions in the absence of protein do not directly produce NO over the time period of the experiment, a requirement if the observed reaction products arise from the direct reaction of NO and protein. These results indicate that the observed products from the reaction of hydroxyurea and oxyHbA, deoxyHbA, and metHbA do not arise from the direct reaction of NO.

The formation of CNmetHb from the reactions of oxy- and deoxyHb with hydroxyurea in the presence of KCN clearly shows that hydroxyurea oxidizes both oxy- and deoxyHb to metHb. The formation of HbCO from the reaction of hydroxyurea and metHb under a CO atmosphere shows that hydroxyurea reduces metHb to deoxyHb. Addition of cyanide to these reactions also completely suppresses HbNO formation as judged by absorption and EPR spectroscopy. These results reveal that hydroxyurea reacts with oxyHbA and deoxyHbA to initially form metHbA and that HbNO arises specifically during the reaction between hydroxyurea and metHbA.

Comparison of the Reaction of Hemoglobin with Hydroxyurea and Hydroxylamine. These reactions of hydroxyurea with hemoglobin display a similarity to the reactions of hydroxylamine (NH_2OH) with hemoglobin. Hydroxylamine reacts with oxyHb (bovine) to produce metHb and small amounts of HbNO (*22*, *23*). EPR measurements of this reaction also show the intermediacy of a nitroxide radical ($NH_2O^{\bullet}$) that yields nitrogen gas and water (*23*). Hydroxylamine reacts with deoxyHb to form metHb and about 0.5 mol of ammonia/mol of deoxyHb oxidized (*22*, *24*). Hydroxylamine also reacts with metHb to form deoxyHb, nitrogen, and small amounts of N_2O (*24*). Under anaerobic conditions, these reactions form a redox cycle between

Scheme 2



deoxyHb and metHb with the hydroxylamine being ultimately converted into ammonia, nitrogen gas, and water (*24*). Absorption measurements show that the reaction of hydroxylamine and deoxy- or metHb does not produce measurable amounts of HbNO, but EPR spectroscopy was not used to analyze these reactions (*24*). The identification of HbNO in our work emphasizes the power of EPR spectroscopy for confirming the presence of small amounts of the paramagnetic hemoglobin iron nitrosyl complex in a mixture.

Scheme 2 depicts the application of this sequence to the reactions of hydroxyurea with oxy-, deoxy-, and metHb. The first and third reactions of Scheme 2 illustrate a proposed redox cycle between Fe^{3+} and Fe^{2+} for the reactions of hydroxyurea with metHb and deoxyHb. Oxidation of hydroxyurea by metHb would produce deoxyHb (Fe^{3+} to Fe^{2+}) and the nitroxide radical **2** (first reaction, Scheme 2). Radical **2** has been proposed to decompose to nitric oxide and observed by EPR spectroscopy during the reaction of hydroxyurea and oxyhemoglobin (second reaction, Scheme 2) (*8*, *10*). Combination of NO produced from the decomposition of **2** with deoxyHb, the other reaction product, forms HbNO (second reaction, Scheme 2) (*26*). Alternatively, NO produced from the decomposition of **2** could possibly form HbNO via the reductive nitrosylation of metHb (*27*). Reduction of the N−O bond of hydroxyurea by 2 equiv of deoxyHb would produce metHb (Fe^{2+} to Fe^{3+}) and urea (third reaction, Scheme 2). Cooxidation of the heme iron and hydroxyurea by the heme-bound oxygen of oxyHb, as previously proposed (*10*), would produce metHb (Fe^{2+} to Fe^{3+}), which could enter the cycle, radical **2**, and hydrogen peroxide (fourth reaction, Scheme 2). The conversion of oxyHb to deoxyHb via metHb by reductants, including phenylhydroxylamine, has also been reported (*28*).

As the formation of CNmetHb from the reactions of oxy- and deoxyHb with hydroxyurea in the presence of KCN clearly shows that hydroxyurea oxidizes both oxy- and deoxyHb to metHb, this model explains the formation of HbNO from the reactions of hydroxyurea with each of these proteins. The addition of KCN also traps metHb as unreactive CNmetHb, which blocks HbNO formation in each of these reactions. In the presence of excess hydroxyurea, the simultaneous occurrence of these three reactions provides an explanation to the kinetic complexities previously observed (*18*, *19*). With the exception of the amount of nitrite produced, sickle cell hemoglobin reacts with hydroxyurea to produce nearly identical products, indicating that the β-6 L-glutamic acid to → L-valine mutation does not generally affect these reactions. At this time, no clear explanation exists as to increased levels of nitrite production observed during the reaction of hydroxyurea with oxy- and deoxyHbS as compared to oxy- and deoxyHbA. Overall, such results support our earlier observation that NO release from hydroxyurea requires only a ferric heme group (*20*).

Reactions of Hydroxyurea and Oxy-, Deoxy-, and Methemoglobin. Addition of hydroxyurea to metHb produces changes in both the absorption and EPR spectra, consistent with the formation of a hydroxyurea−metHb complex (*10*, *18*). Inner-sphere electron transfer within such a complex would produce deoxyHb and the nitroxide radical (**2**). The inability of CNmetHb to react with hydroxyurea also strongly suggests that this reaction proceeds through an inner-sphere mechanism. The deoxyHb could react with NO formed from the decomposition of **2** to give HbNO, with oxygen from the air to form oxyHb, or with hydroxyurea to form metHb. Under a CO atmosphere, deoxyHb would also react with CO to form HbCO, and the identification of HbCO from this reaction clearly shows that hydroxyurea reduces metHb. Identification of HbNO from the reaction of metHb and hydroxyurea under a CO atmosphere indicates that CO and NO compete for the ferrous heme iron under these conditions.

The inability of HbCO to react with hydroxyurea also suggests that the reaction of deoxyHb and hydroxyurea occurs through an inner-sphere mechanism (*19*). Urea production from hydroxyurea requires a two-electron reduction of the N−O bond and by analogy to the reaction of deoxyHb and hydroxylamine would require 2 equiv of ferrous heme (*24*). At this time, structural information on discrete intermediates of this reaction does not exist. The metHb formed in the reaction of hydroxyurea and deoxyHb would be able to react with hydroxyurea to form deoxyHb and complete the redox cycle. In the presence of KCN, metHb would react to form CNmetHb.

Absorption spectroscopy shows the formation of metHb from the reaction of oxyHb and hydroxyurea, and previous EPR studies indicate the presence of the nitroxide radical (**2**) in this reaction (*10*, *18*). The metHb formed in this reaction would be able to react with hydroxyurea to form deoxyHb and enter the redox cycle leading to HbNO. In the presence of KCN, metHb would react to form CNmetHb. The production of significant amounts of nitrate only during the reaction of oxyHb with hydroxyurea provides evidence for the involvement of NO, which rapidly reacts with oxyHb to form metHb and nitrate, in these reactions (*29*). At this time, these results must be cautiously interpreted as the final product stoichiometries have yet to be rigorously established and as nitrate could arise from other pathways including the oxidation of nitrite or the reaction of nitroxyl (HNO) with oxyHb (*30*). Failure to detect N_2O during the reactions of hydroxyurea and hemoglobin argues against the involvement of HNO. However, the rapid reaction of HNO with metHb to form HbNO could explain the lack of observed N_2O and presents another alternative pathway for HbNO formation (*30*). These results highlight the importance of understanding the chemical properties including the formation, stability, and NO-donating ability of the nitroxide radical (**2**).

Formation of metHb and the nitroxide radical (**2**) from the cooxidation of oxyHb and hydroxyurea also should produce 1 equiv of hydrogen peroxide. Addition of hydrogen peroxide to the reaction of oxyHb and hydroxyurea increases the rate of the reaction but does not alter the protein product composition (*20*). Hydrogen peroxide can oxidize methemoglobin to a reactive ferrylhemoglobin species (*31*). A previous chemiluminescence study provides evidence for a ferrylhemoglobin intermediate in the reaction of hydroxylamine and oxyHb, but the presence of this species in the reaction of hydroxyurea and oxyHb is not clear (*32*). Addition of catalase to the reaction of hydroxyurea and oxyHb does not alter the rate or observed products, indicating that hydrogen peroxide either does not play a role in these reactions or is never released from the iron heme. Further work will be required to establish the intermediacy of ferrylhemoglobin in these reactions.

Reactions of Hydroxyurea Derivatives and Hemoglobin. Experiments with the hydroxyurea derivatives **3** and **4** provide further information regarding the reaction mechanism and the structural requirements of NO release. The inability of **3** to react with oxyHb and metHb indicates that these reactions require an oxidizable *N*-hydroxy group. Scheme 2 predicts these results, which support previous work showing that urea does not oxidize oxyHb (*18*). The failure of **3** to react with deoxyHb also indicates that hydroxyurea must react with deoxyHb by an inner-sphere mechanism where hydroxyurea directly binds to the iron atom through the *N*-hydroxyl group. The *O*-methyl group of **3** prevents the association and reaction of **3** with the heme iron.

Scheme 2 predicts derivative **4**, which possesses an oxidizable *N*-hydroxy group, will react with both oxy- and metHb and presumably form a nitroxide radical. Absorption measurements clearly show that **4** oxidizes oxyHb to metHb, and the formation of HbCO from the reaction of **4** with metHb under a CO atmosphere shows that hydroxyurea reduces metHb to deoxyHb. However, the failure to detect HbNO indicates that **4** or any radicals derived from it cannot transfer NO during these reactions. The absence of nitrate formation in the reaction of **4** with oxyHb also supports the direct involvement of NO in the reactions of hydroxyurea. The lack of NO transfer in these reactions is most likely related to the fact that formation of NO from **4** requires C−N bond cleavage. Unlike **3**, the free *N*-hydroxy group allows **4** to coordinate and react with deoxyHb. These results also support the idea that the observed protein products from the reaction of hydroxyurea and these hemoglobins result from reactions between these proteins and hydroxyurea as opposed to direct reactions with NO. Taken together, the results from experiments with **3** and **4** indicate that NO transfer during the reactions of hydroxyurea and hemoglobin requires a free unsubstituted acylhydroxylamine (−CONHOH) group.

Potential Biological Significance. These reactions of hydroxyurea and hemoglobin that form metHb and small amounts of HbNO could have important implications to sickle cell disease treatment. While the success of hydroxyurea has generally been attributed to an increase in fetal hemoglobin production, such a mechanism does not exclude a role played by HbNO and metHb formed from the reaction of hydroxyurea and hemoglobin. As small decreases in the concentration of deoxyHb can greatly increase the delay time of protein polymerization (*33*), the formation of even small amounts of HbNO and metHb from the reaction of hydroxyurea and deoxyHb could benefit patients by increasing the delay time for polymerization. The formation of metHb from these reactions could lead to a number of deleterious effects on the sickle cell patient, which have been reviewed (*34*). However, the treatment of rats or whole red blood cells with hydroxyurea fails to produce significant amounts of metHb, suggesting that methemoglobin reductases, not present in the in vitro experiments, convert metHb to the ferrous form (*11*, *35*).

The formation of HbNO (2−6% of the total protein) from the reaction of sickle cell hemoglobin with hydroxyurea could also play an important role in the beneficial effects of hydroxyurea treatment of sickle cell disease. 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 show that the NO derives from hydroxyurea (*11*, *12*). Formation of small amounts of HbNO may lead to decreased polymerization as partial iron nitrosylation may form hemoglobins with improved solubility properties that also retain the ability to function as oxygen carriers capable of oxygen delivery under physiological conditions (*16*, *36*). Recent work also indicates that HbNO may transport and release nitric oxide through the intermediacy of *S*-nitrosohemoglobin (*37*). The in vivo release of even nanomolar amounts of NO derived from the small amount of HbNO formed from the reaction of hydroxyurea and hemoglobin would provide beneficial effects by increasing microvascular blood flow. 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 (*13*, *14*). In addition to the important roles that *S*-nitrosohemoglobin plays in blood pressure control by releasing NO at low oxygen pressures (*38*, *39*), 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 (*13*, *40*, *41*). While these investigations failed to establish the in vitro formation of *S*-nitrosohemoglobin from these reactions, again the in vivo formation of even nanomolar amounts of *S*-nitrosohemoglobin may provide beneficial effects by increasing microvascular blood flow through NO release or stabilizing the R-state of the protein.

In summary, hydroxyurea reacts with oxy-, deoxy, and metHb to form HbNO with the NO group deriving from the −NHOH group of hydroxyurea. Whether in vivo nitric oxide production occurs from the reaction of hydroxyurea and hemoglobin remains to be defined. The in vitro reactions of hydroxyurea and hemoglobin occur at modest rates as evidenced by the large excess of hydroxyurea used in these studies (*18*, *19*, *20*). The in vivo reactions may be accelerated by the presence of hydrogen peroxide or organic phosphates, which have been shown to modulate the rate of reaction of hydroxylamine and hemoglobin (*22*). Alternatively, hydroxyurea could rapidly react with some oxidative enzyme, such as a peroxidase or oxygenase, to release NO that is subsequently trapped by hemoglobin to form HbNO. In any event, these studies define the reactivity of hydroxyurea with hemoglobin and demonstrate that hydroxyurea can liberate NO in its reactions with heme proteins. Such information will be applied to the design of new hydroxyurea-derived

nitric oxide releasing agents that may be of ultimate therapeutic use in sickle cell disease.

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