Nitrosylation of Sickle Cell Hemoglobin by Hydroxyurea

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Hydroxyurea (**1**) represents a new treatment for sickle cell anemia,[2] a disease that affects about one out of 600 people of African descent born in the United States.[3] While some of the beneficial effects of hydroxyurea treatment appear to result from an increase in the production of fetal hemoglobin, some patients benefit from hydroxyurea treatment before their levels of fetal hemoglobin increase, indicating that the positive effects of this drug cannot be completely explained by the increase of this protein and suggesting other mechanisms of action.[4] The detailed molecular mechanism of hydroxyurea's action remains poorly described, and speculation regarding the possible involvement of nitric oxide (NO) in the biological actions of hydroxyurea exists.[5] Identification of the characteristic decomposition products of NO during the oxidation of hydroxyurea with various combinations of oxidants supports this suggestion.[5]

Both NO and nitrosyl hemoglobin (HbNO, Fe^{2+}−NO), which forms as a minor product upon reaction of hydroxyurea with oxyhemoglobin (HbO_2, Fe^{2+}, Scheme 1),[6,7] have recently been proposed as potential therapies for sickle cell disease.[8] The identification of HbNO from hydroxyurea-treated rats also provides evidence for the in vivo metabolic production of NO from hydroxyurea.[9] Under physiological conditions, HbNO exists in an oxygen-dependent equilibrium with *S*-nitrosohemoglobin (S-nitrosylated on the β-93 cysteine residues), a vasorelaxant protein whose formation could benefit sickle cell patients.[10] Here, we report for the first time that the in vitro reaction of sickle cell oxyhemoglobin with hydroxyurea to form sickle cell nitrosylhemoglobin involves the specific transfer of NO from the NHOH group of the drug (Scheme 1).

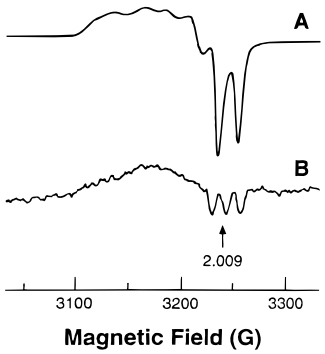
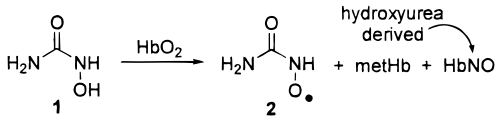


Figure 1. EPR spectra of $Hb^{15}NO$ (trace A) and $Hb^{14}NO$ (trace B). The spectra are of samples in a liquid nitrogen Dewar observed at 9.1 GHz. The 2.009 *g* value arrow is at the center of the nitrogen hyperfine patterns. The hyperfine constants are 25 G for ^{15}N and 17.5 G for ^{14}N.

Scheme 1



Evidence that the NO group from HbNO derives from the NH−OH group of hydroxyurea was obtained by the use of ^{15}N-labeled hydroxyurea and observance of the isotope effect on the hyperfine splitting pattern of the EPR spectrum of HbNO. ^{15}N-Hydroxyurea (0.76 M), prepared by the condensation of commercially available ^{15}N-hydroxylamine and trimethylsilyl (TMS) isocyanate, was incubated at room temperature with sickle cell oxyhemoglobin (0.42 mM) in 0.1 M sodium phosphate buffer (pH 7.3). After 20 h, a distinct visible absorbance change indicated the primary conversion of HbO_2 to methemoglobin (metHb, Fe^{3+}, Scheme 1), and the presence of HbNO was confirmed by EPR spectroscopy (77 K).[6,7] Addition of sodium dodecyl sulfate (40 mM final concentration) prior to sample freezing cleanly resolved the hyperfine splitting patterns, allowing for analysis of the nitrogen coupling. The EPR spectrum of the reaction of sickle cell HbO_2 and ^{15}N-hydroxyurea after denaturation displayed a two peak pattern (Figure 1, trace A) consistent with electronic coupling to ^{15}N ($I = 1/2$) and identical to a reported $Hb^{15}NO$ EPR spectrum.[11] In contrast, the EPR spectrum of the HbNO formed by the reaction of sickle cell HbO_2 and ^{14}N-hydroxyurea demonstrated a three-peak pattern (Figure 1, trace B) consistent with electronic coupling to ^{14}N ($I = 1$). These results indicate that the NO group of the HbNO formed in the reaction of HbO_2 and hydroxyurea specifically derives from the NHOH portion of hydroxyurea.

Model oxidation of hydroxyurea with hydrogen peroxide followed byproduct analysis indicates that hydroxyurea decomposes with the formation of nitroxyl (HNO) and nitric oxide (NO). As indicated by EPR spectroscopy,[6,12] these conditions produce the same nitroxide radical (**2**, Scheme 1) as the HbO_2 oxidation of hydroxyurea in the absence of protein, which could complicate the detection of any reactive species formed. The hydrogen peroxide oxidation of hydroxyurea (1.0 equivalent) produced nitrous oxide (N_2O, 17%) as the major nitrogen oxide, carbon dioxide (CO_2, 22%), ammonia (NH_3, 25%), and nitrite (NO_2^-, 6%) after 24 h.[13]

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(13) Full details of these procedures can be found in the Supporting Information.

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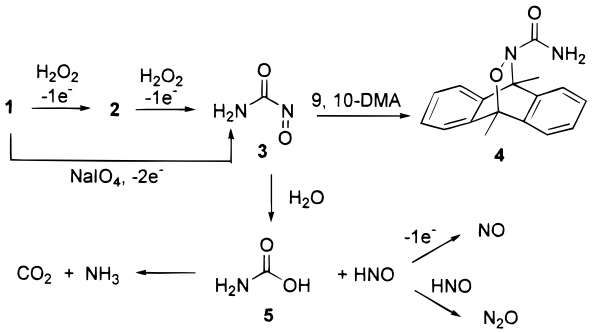
Scheme 2

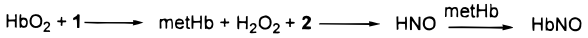


These yields are based upon the possibility of one molecule of hydroxyurea producing one molecule each of CO_2, NH_3, and nitrogen monoxide (NO or HNO). These products could not be detected after 1 h of reaction. Nitrous oxide identification provides strong evidence for the intermediacy of nitroxyl (HNO), which rapidly dimerizes and dehydrates to form N_2O.[14] Gas-phase infrared spectroscopic analysis of the N−N bond stretching frequency indicated the formation of only $^{15}N^{15}NO$ from the hydrogen peroxide oxidation of ^{15}N-hydroxyurea, establishing that both nitrogen atoms of the produced N_2O are derived from the NH−OH group of hydroxyurea.[15] Nitrite formation generally provides evidence for the involvement of NO.[16] The yields of the observed products indicate the initial formation of nearly equal amounts of CO_2, NH_3, and nitrogen monoxides (HNO or NO), as judged by the sum of the yields of N_2O and NO_2^- from hydroxyurea. Increasing the amount of hydrogen peroxide (2.0 equiv) increased the amount of CO_2 (44%), NH_3 (48%), and nitrogen oxides formed (N_2O, 29%, NO_2^-, 29%), with the yield of NO_2^- apparently increasing at the expense of N_2O formation.

Additional evidence relevant to the mechanism of hydroxyurea oxidation includes the trapping of a *C*-nitrosoformamide intermediate. Oxidation of hydroxyurea with hydrogen peroxide (1.3 equiv, $CH_2Cl_2/CH_3OH/H_2O$) in the presence of 9,10-dimethylanthracene (9,10-DMA) produced a cycloadduct (**4**, 28%, Scheme 2).[17] The identification of this cycloadduct provides clear evidence for the involvement of the *C*-nitrosoformamide (**3**), which has previously been trapped by 9,10-DMA after periodate oxidation of hydroxyurea.[18] Aqueous sodium periodate oxidation (1.1 equiv) of hydroxyurea in the absence of 9,10-DMA rapidly produced (1 h) nitrous oxide (65%), carbon dioxide (59%), ammonia (58%), and nitrite (10−15%).

The mechanism depicted in Scheme 2 has been proposed to explain these results regarding the chemical oxidation of hydroxyurea. Hydrogen peroxide oxidation of hydroxyurea

Scheme 3



proceeds through two one-electron oxidations, possibly catalyzed by adventitious metal ions, via the nitroxide radical (**2**, observed by EPR)[12] to produce **3**, which reacts with 9,-10-DMA to produce **4** or rapidly hydrolyzes in the absence of 9,10-DMA to carbamic acid (**5**) and HNO. Carbamic acid (**5**) decomposes to carbon dioxide and ammonia. Nitrous oxide forms from the rapid dimerization ($k = 2-8 \times 10^9\ M^{-1}\ s^{-1}$) and dehydration of nitroxyl.[14] Peroxide oxidation of HNO to nitric oxide ($E_{NO/NO}{}^{-\circ} = -0.33V$) could explain the formation of nitrite. Increasing the amount of hydrogen peroxide increases NO_2^- formation at the expense of N_2O production, suggesting that under these oxidative conditions nitric oxide and ultimately nitrite formation arise from further HNO oxidation.[19] With sodium periodate, the formation of **3** from hydroxyurea occurs directly through a rapid two-electron oxidation, resulting in increased yields of the hydrolysis products of **3** compared to the slower stepwise peroxide-mediated oxidation.

These results and the EPR spectroscopic identification of **2** during both the hydrogen peroxide and HbO_2 oxidation of hydroxyurea indicate the potential involvement of nitroxyl (HNO) and *C*-nitrosoformamide (**3**) in the HbO_2 reaction with hydroxyurea. The co-oxidation of the heme iron (Fe^{2+} to Fe^{3+}) and hydroxyurea to **2** by heme-bound oxygen with the formation of hydrogen peroxide has been suggested as the initial step in the reaction of hydroxyurea and HbO_2 (Scheme 3).[6] On the basis of our model results, hydrogen peroxide generation during this reaction provides a mechanism for nitroxyl production through the oxidation of hydroxyurea or **2** to **3** and subsequent hydrolysis. The reductive nitrosylation of metHb, the major hemoglobin reaction product, by HNO provides a plausible explanation for HbNO production (Scheme 3).[20] At this time, other alternative less direct mechanisms of HbNO formation, including the reaction of nitric oxide with metHb,[21] the reaction of nitric oxide with deoxyhemoglobin (Fe^{2+}),[22] and nitroxyl formation through a metHb-catalyzed peroxidase-like oxidation of hydroxyurea followed by reductive nitrosylation, must also be considered.[23]

Given the possible use of NO as a sickle cell disease therapy and the indications of alternative mechanisms for the beneficial effects of hydroxyurea treatment, the identification of HbNO from the reaction of sickle cell oxyhemoglobin and hydroxyurea with the NO group being derived from hydroxyurea could be physiologically significant. Further experiments to better understand the mechanism of NO transfer and to determine whether this transfer of NO is biologically relevant are planned.

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Supporting Information Available: Full experimental details including the synthesis and characterization of ^{15}N-hydroxyurea, gas-phase infrared spectroscopic data, and the X-ray crystallographic data of **4** (15 pages).

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