ELSEVIER

Biophysical Chemistry 84 (2000) 1–11

Biophysical Chemistry

www.elsevier.nl/locate/bpc

The reactions of myoglobin, normal adult hemoglobin, sickle cell hemoglobin and hemin with hydroxyurea

Jeremy W. Rupon[a], Shirely R. Domingo[b], Sara V. Smith[b], Bharat K. Gummadi[b], Howard Shields[b], Samir K. Ballas[c], S. Bruce King[a,1], Daniel B. Kim-Shapiro[b,*]

[a] *Department of Chemistry, Wake Forest University, Winston-Salem, NC 27103, USA*
[b] *Department of Physics, Wake Forest University, Winston-Salem, NC 27103, USA*
[c] *Department of Medicine, The Cardeza Foundation, Jefferson Medical College, Philadelphia, PA, USA*

Received 16 August 1999; received in revised form 25 October 1999; accepted 25 October 1999

Abstract

The kinetics of the reaction of hydroxyurea (HU) with myoglobin (Mb), hemin, sickle cell hemoglobin (HbS), and normal adult hemoglobin (HbA) were determined using optical absorption spectroscopy as a function of time, wavelength, and temperature. Each reaction appeared to follow pseudo-first order kinetics. Electron paramagnetic resonance spectroscopy (EPR) experiments indicated that each reaction produced an FeNO product. Reactions of hemin and the ferric forms of HbA, HbS, and myoglobin with HU also formed the NO adduct. The formation of methemoglobin and nitric oxide–hemoglobin from these reactions may provide further insight into the mechanism of how HU benefits sickle cell patients.

Keywords: Sickle cell hemoglobin; Hydroxyurea; Methemoglobin; Hemin; Nitric oxide; Time resolved absorption spectroscopy

* Corresponding author. Tel.: +1-336-758-5774; fax: +1-336-758-4656.
E-mail addresses: kingsb@wfu.edu (S. Bruce King), shapiro@wfu.edu (D.B. Kim-Shapiro)
[1] Co-corresponding author. Tel.: +1-336-758-4993; fax: +1-336-758-6142.

0301-4622/00/$ - see front matter
PII: S0301-4622(99)00132-5

1. Introduction

Hydroxyurea (HU) has recently been used to treat sickle cell disease, a condition that affects one out of every 600 African Americans [1–3]. A single mutation in the amino acid sequence of hemoglobin (β6 L-glutamic acid → L-valine) causes this disease [4]. Under hypoxic conditions, this mutation allows sickle cell hemoglobin (HbS) to polymerize causing distortion and increased rigidity of the sickle red blood cell. These rigid cells contribute to microvascular occlusion resulting in tissue eschemia, organ damage, pain and death [5].

Hydroxyurea benefits sickle cell patients by increasing the level of fetal hemoglobin (HbF), which reduces the tendency of HbS to polymerize [1,2]. The results of a multiparameter study of sickle erythrocytes from patients undergoing hydroxyurea therapy indicate that an increase in the proportion of F-cells (cells containing approx. 20% HbF) is the primary effect of hydroxyurea [6]. However, clinical improvement and increased levels of HbF have not always occurred simultaneously [7]. These findings suggest that other mechanisms may be responsible for a portion of the improvement of sickle cell patients treated with hydroxyurea [1,2]. Previous explanations of these beneficial effects include a proposal that hydroxyurea increases mean cell volume (MCV) and reduces neutrophil count [2]. We have proposed that hydroxyurea's benefits partially arise from its reaction with oxyhemoglobin (HbO_2) to form methemoglobin (metHb) and nitrosyl hemoglobin (HbNO) [8–10]. Nitric oxide (NO) may also be involved in the reaction of oxyhemoglobin and HU [10,11]. HbNO reportedly exists in an oxygen-dependent equilibrium with S-nitrosohemoglobin, a vasoactive protein S-nitrosated at the β-93 residue [12]. The β-93 cysteine residues of hemoglobin have also previously been implicated in the reactions of nitrogen monoxides with heme proteins [13]. To determine the importance of protein structure (specifically the β-93 cysteine) in the reaction of HU with hemoglobin, we have examined the reaction of HU with oxyHbA, oxyHbS, oxyMb, and hemin. We wish to report the results regarding both the kinetics and product distribution of these reactions.

2. Experimental

Oxymyoglobin (MbO_2) was prepared from equine metmyoglobin (metMb, Sigma Chemical Company). A sample of metMb (50 mg, 3.13 μmol) was placed in a sealed flask and flushed with argon for 30 min. Deoxygenated buffer [0.1 M sodium phosphate (pH 7.3) 1.75 ml] was transferred into the flask with stirring to form a metMb solution. Sodium dithionite (22 mg, 127 μmol, Sigma) was added, and the color of the solution went from brown to dark red. The solution was purified through a pre-packed column of G-25 sephadex (Supleco, Bellfonte, PA) to remove excess dithionite, and diluted with buffer until the concentration was between 250 and 300 μM.

Excess, discarded blood was obtained that had

Table 1

Hydroxyurea (mM)	Apparent rate constant (s^{-1})	Error (±)	Bimolecular rate constant (s^{-1} M^{-1})	Avg. bimolecular rate constant
Myoglobin 37°C				
660	1.4×10^{-3}	2.6×10^{-5}	2.2×10^{-3}	$2.5 \pm 0.4 \times 10^{-3}$
500	1.2×10^{-3}	2.9×10^{-5}	2.3×10^{-3}	
330	1.0×10^{-3}	3.7×10^{-5}	3.0×10^{-3}	
Room temperature				
660	6.8×10^{-4}	2.0×10^{-5}	1.0×10^{-3}	$9.5 \pm 2.0 \times 10^{-4}$
500	3.8×10^{-4}	1.4×10^{-5}	7.5×10^{-4}	
330	3.5×10^{-4}	1.2×10^{-5}	1.1×10^{-3}	

Table 2

Hydroxyurea (Mm)	Apparent rate constant (s^{-1})	Error (±)	Bimolecular (s^{-1} M^{-1})	Avg. bimolecular
HbA 37°C				
660	6.2×10^{-4}	1.9×10^{-5}	9.3×10^{-4}	$6.8 \pm 2.1 \times 10^{-4}$
(β-93 Blocked) 660	4.7×10^{-4}	4.5×10^{-5}	7.1×10^{-4}	
500	2.9×10^{-4}	3.5×10^{-5}	5.7×10^{-4}	
(H_2O_2) 500	7.7×10^{-4}	9.6×10^{-5}	1.5×10^{-3}	
330	1.8×10^{-4}	2.5×10^{-5}	5.5×10^{-4}	
Room temperature				
660	9.9×10^{-5}	1.1×10^{-6}	1.5×10^{-4}	$1.3 \pm 0.2 \times 10^{-4}$
500	5.9×10^{-5}	8.3×10^{-7}	1.2×10^{-4}	
330	3.6×10^{-5}	2.1×10^{-6}	1.1×10^{-4}	

been drawn from patients homozygous in the gene responsible for sickle cell disease with low (less than 5%) fetal hemoglobin following federal regulations and guidelines. Hemoglobin was prepared as described previously [14]. 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 0.1 M sodium phosphate buffer, pH 7.3. No difference was previously observed in reaction HU with purified deoxy-HbS and the hemolytsate [15]. The hemoglobin sample was pelleted in liquid nitrogen and stored at −80°C. Samples used for spectrophotometric measurements were thawed and used within 24 h. The thawed hemoglobin was diluted with phosphate buffer until the concentration was between 250 μM and 300 μM. HbA was prepared in a similar fashion. The β-93 cysteines of 300 μM oxyHbA were blocked by reaction with a 3 M excess of iodoactemide (Aldrich) for 3 h in the dark. The solution was purified by a Sephadex G-25 column, and the method of Ellman was used to confirm blocking [16].

Hemin (Sigma) solutions for kinetic and EPR measurements were made from a stock solution in ethylene glycol (4.5 mM) with a drop of ammonium hydroxide (14.8 N). The hemin stock solution was diluted with ethylene glycol for spectroscopic measurements at 250–300 μM.

A Hewlett Packard (HP) 8452A diode array spectrophotometer (equipped with a heating jacket) and a Perkin-Elmer (PE) Lambda 9 spectrophotometer were used to obtain the kinetic data. Cuvettes (0.5 cm and 0.2 cm) were used in the HP and PE spectrophotometers, respectively. Three different concentrations of HU were used in the experiments (1.32 M, 1.0 M, and 0.66 M) and reactants were mixed in the cuvettes in a 1:1 volumetric ratio. For the measurements of oxyMb,

Table 3

Hydroxyurea (mM)	Apparent rate constant (s^{-1})	Error (±)	Bimolecular (s^{-1} M^{-1})	Avg. bimolecular
HbS 37°C				
660	4.3×10^{-4}	2.5×10^{-5}	6.4×10^{-4}	$4.9 \pm 1.7 \times 10^{-4}$
500	2.6×10^{-4}	1.6×10^{-5}	5.2×10^{-4}	
330	1.0×10^{-4}	1.6×10^{-5}	3.1×10^{-4}	
Room temperature				
660	1.5×10^{-4}	5.3×10^{-5}	2.3×10^{-4}	$2.0 \pm 0.4 \times 10^{-4}$
500	1.1×10^{-4}	3.6×10^{-5}	2.2×10^{-4}	
330	5.1×10^{-5}	2.0×10^{-5}	1.6×10^{-4}	

oxyHbA, and oxyHbS at 37°C, data was collected for 1 h every 100 s. At room temperature, data was collected for 12 h every 250 s. The blocked oxyHbA reacted with 1.32 M HU in a 1:1 volumetric ratio at 37°C for 1 h, and scans were taken every minute. For hemin at 37°C, data was collected every 90 s for 4 h and every 300 s for 12 h at room temperature. Hydroxyurea was dissolved in ethylene glycol for the hemin reactions. Three trials were performed with each concentration of HU at each temperature. The kinetic data was analyzed using Specfit (Spectrum Software Associates, Chapel Hill, NC) using singular value decomposition (SVD) and global analysis.

Electron paramagnetic resonance spectroscopy (EPR) samples for oxyMb, oxyHbA, and oxyHbS were prepared by reacting the oxygenated proteins (1 mM) with 1.32 M HU in a 1:1 volumetric ratio for 12 h. Blocked HbA (2.2 mM) reacted with 1.32 M HU in a 1:1 volumetric ratio for 12 h. Argon de-gassed hemin (2.2 mM) in ethylene glycol was reacted with both [^{14}N]- and [^{15}N]hydroxyurea (0.433 M) in de-gassed ethylene glycol in a 1:1 volumetric ratio. [^{15}N]hydroxyurea was prepared as previously described [10]. These solutions were transferred to an EPR tube and frozen in liquid nitrogen (77 K). EPR spectra were taken on a Varian E-line Spectrometer using 5-mW microwave power, 5-G modulation amplitude and 9.1-GHz microwave frequency.

3. Results

Oxy(Mb, HbA, and HbS) display characteristic absorbances at 540 and 577 nm. When each protein reacted with HU at 37°C and room temperature, these absorbances decreased with time and absorbances at 635 and 500 nm increased indicating the formation of met(Mb, HbA, and HbS). Figs. 1–3 depict typical spectra for the reaction of HU with oxyMb, oxyHbA, and oxyHbS, respectively. The final reaction products resemble that which we reported earlier for oxyHbS at room temperature [9]. Spectral analysis of that reaction indicated the presence of HbNO, metHbS, a Hb-HU adduct and hemichromes. Close examination of the spectra indicates that there are no perfect isosbestic points. As discussed previously in the case of oxyHbS [9], the lack of an isosbestic is most likely due to the formation of other species besides the met forms: the NO adducts and

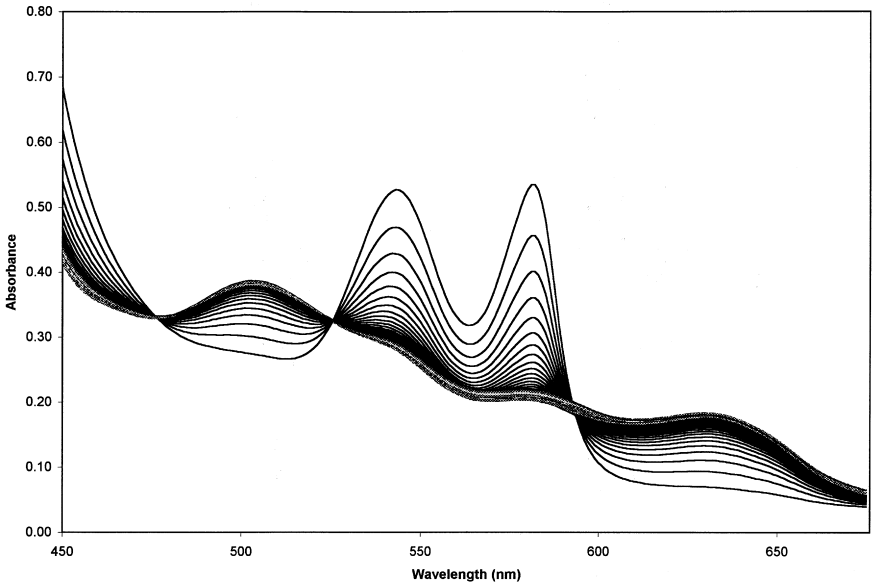

Fig. 1. Time resolved absorption of the reaction of hydroxyurea (660 mM) and oxyMb (270 μM) at room temperature. Measurements are shown for 10-min intervals for 12 h.

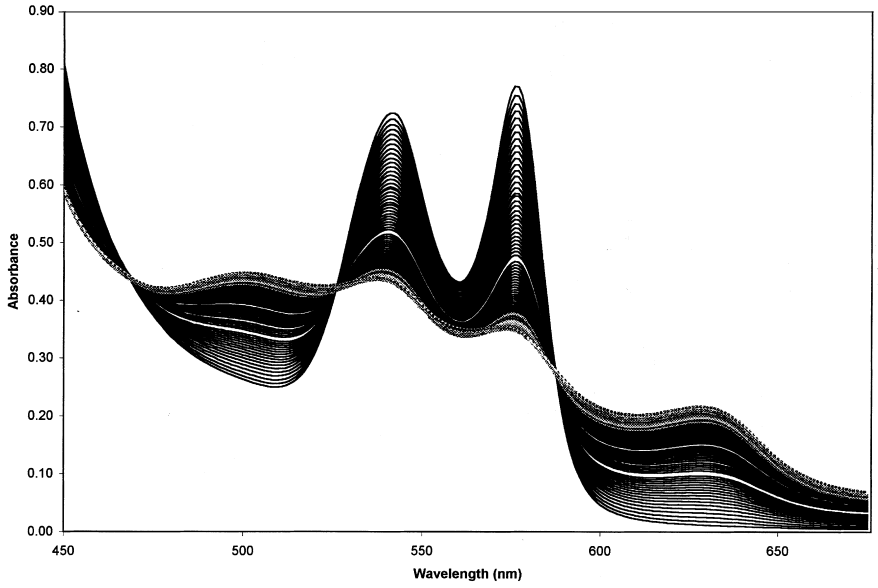

Fig. 2. Time resolved absorption of the reaction of hydroxyurea (660 mM) with oxyHbA (270 μM) at room temperature. Measurements are shown for 10-min intervals for 12 h.

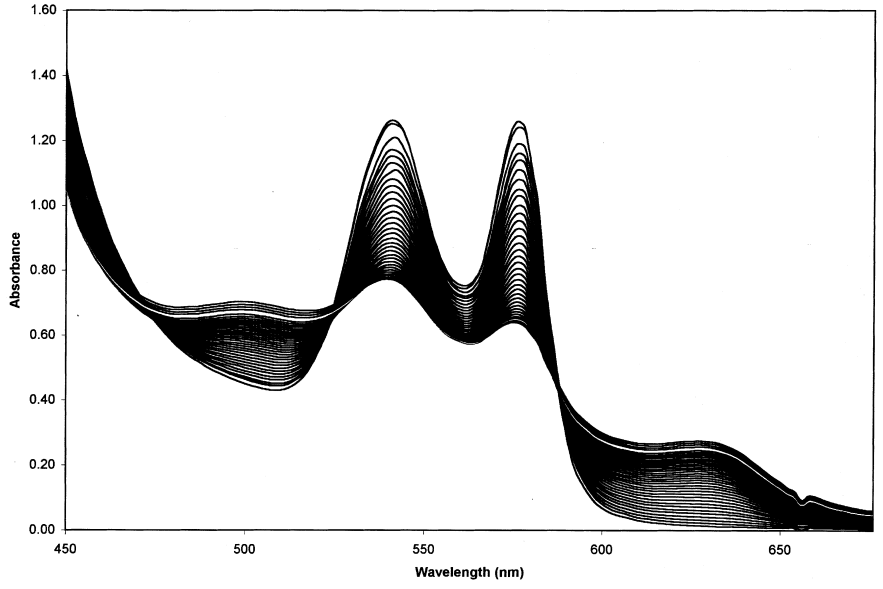

Fig. 3. Time resolved absorption of the reaction of hydroxyurea (660 mM) and oxyHbS (270 μM) at 37°C. Measurements are shown for 100-s intervals for 1 h. The dip in the spectrum at 655 nm is the result of instrumental error.

[1]Rate constants at each concentration are averages of three trials. Bimolecular rate constants were determined by dividing these average rate constants by the concentration of HU used. Standard deviations were determined using the $n-1$ method.

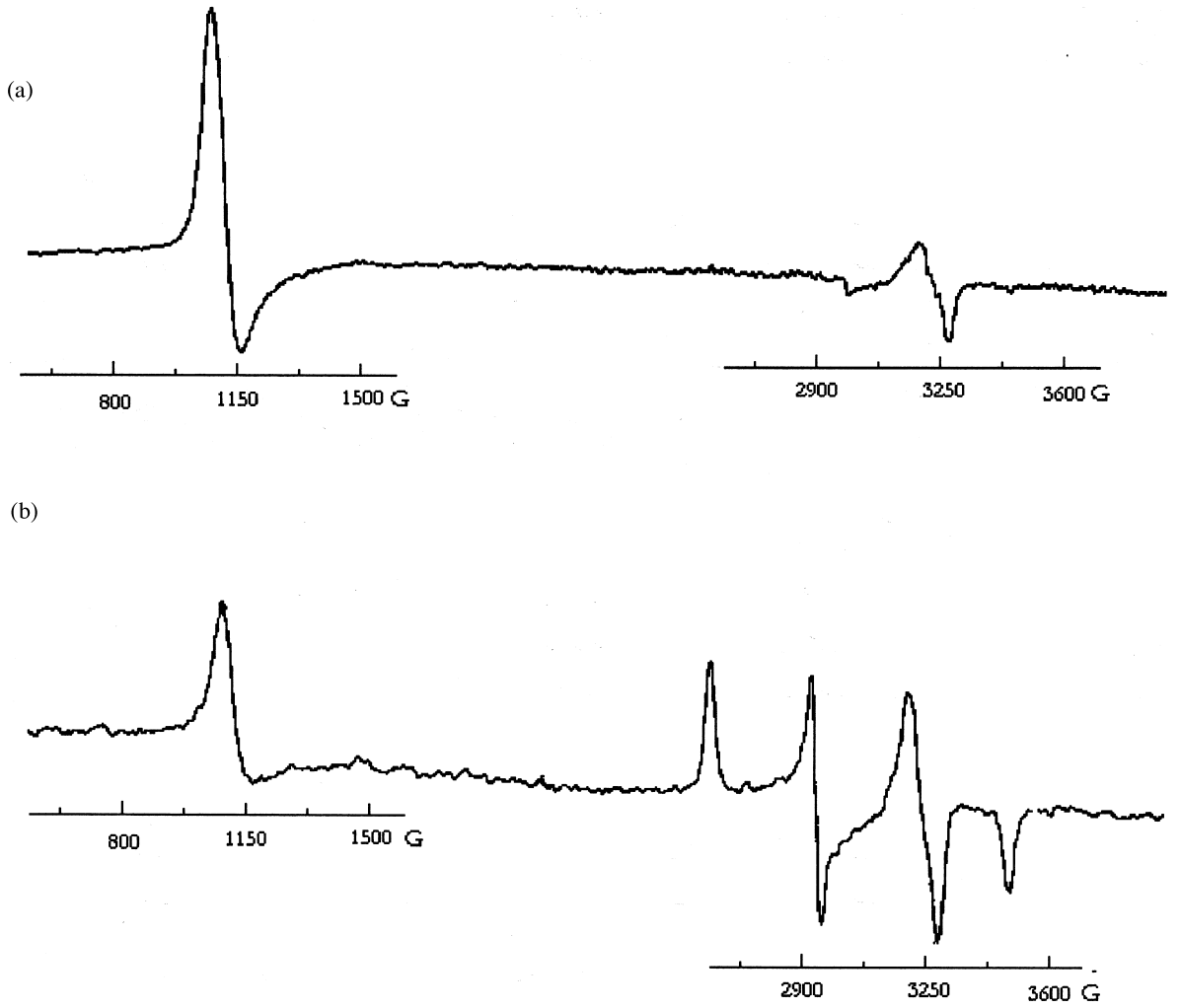

Fig. 4. EPR of the products of oxyMb + HU (a) and oxyHbA + HU (b). The products of oxyHbS and HU have previously been shown [9]. The protein concentration was 1 mM and the HU concentration was 660 mM. The $g = 2$ signal corresponds to a field of 3250 G, while the $g = 6$ signal corresponds to a field of 1083 G for both spectra.

Table 4

Hydroxyurea (mM)	Apparent rate constant (s^{-1})	Error (±)	Bimolecular (s^{-1} M^{-1})	Avg. Bimol.
Hemin 37°C				
660	2.8×10^{-4}	7.4×10^{-6}	4.3×10^{-4}	$2.7 \pm 1.4 \times 10^{-4}$
500	1.1×10^{-4}	5.1×10^{-6}	2.1×10^{-4}	
330	5.3×10^{-5}	4.3×10^{-6}	1.6×10^{-4}	
Room temperature				
660	1.4×10^{-4}	2.7×10^{-6}	2.2×10^{-4}	$1.9 \pm 0.4 \times 10^{-4}$
500	9.8×10^{-5}	2.5×10^{-6}	2.0×10^{-4}	
330	5.0×10^{-5}	1.8×10^{-6}	1.5×10^{-4}	

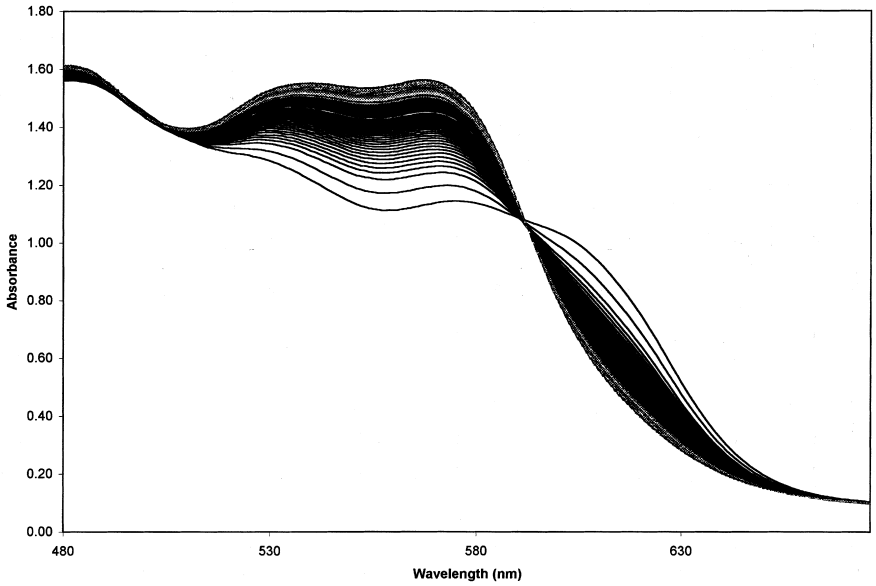

Fig. 5. Time resolved absorption of the reaction of hydroxyurea (660 mM) with hemin (270 μM) at room temperature. Measurements are shown for 300-s intervals for 12 h.

hemichromes. The presence of hemichromes is evidenced by increased turbidity of the sample. Tables 1–3 summarize the kinetic data for both the 37°C and room temperature reactions.[1] The kinetic data for the room temperature reaction of oxyHbS and HU has previously been reported [9]. Experiments with β-93 cysteine-blocked HbA demonstrated a similar absorbance change and rate (Table 2).

EPR spectroscopic experiments confirmed the presence of MbNO and HbA-NO as well as the met-forms of these proteins (Fig. 4). The formation of HbS-NO from the reaction of oxyHbS and HU has previously been shown [10]. Trace A (Fig. 4) shows a peak at $g = 6$ (1083 G) characteristic of the high spin Fe^{3+} in metMb, and a peak at $g = 2$ (3250 G) characteristic of MbNO. No splitting is seen in the $g = 2$ MbNO signal, which is also characteristic of the Mb protein. Trace B (Fig. 4) confirms the formation of HbA-NO from the reaction of oxyHbA with HU. Both spectra also show the three characteristic low-spin ferric peaks around $g = 2$ (2600, 2900, and 3450 G). Experiments with β-93 cysteine-blocked HbA also demonstrated the formation of HbA-NO.

Hydroxyurea also reacted with hemin (Fe^{3+}) and Fig. 5 depicts the change in absorption for this reaction over time. The absorbance rises between 500 and 600 nm and decreases between 600 and 650 nm resulting in an isobestic point at 600 nm. Table 4 summarizes the kinetic data for this reaction at various HU concentrations and temperatures.

EPR spectroscopy indicates the formation of a hemin-NO complex during the reaction of hemin and HU. The EPR spectrum of hemin treated with [^{15}N]HU produced a two peak pattern consistent with electronic coupling to ^{15}N ($I = 1/2$, Fig. 6, Trace A) [17,18]. The reaction with [^{14}N]HU produced a three peak pattern consistent with the electronic coupling to ^{14}N ($I = 1$, Fig. 6, Trace B). These results demonstrate that the NO of the hemin-NO complex is derived from the −NHOH group of HU. Reactions between reduced hemin (Fe^{2+}) and hydroxyurea demonstrate a rapid oxidation of hemin in an oxygen free environment (data not shown).

4. Discussion

The time resolved spectra of the reaction of hydroxyurea with oxyMb, oxyHbA, and oxyHbS demonstrate the formation of the corresponding

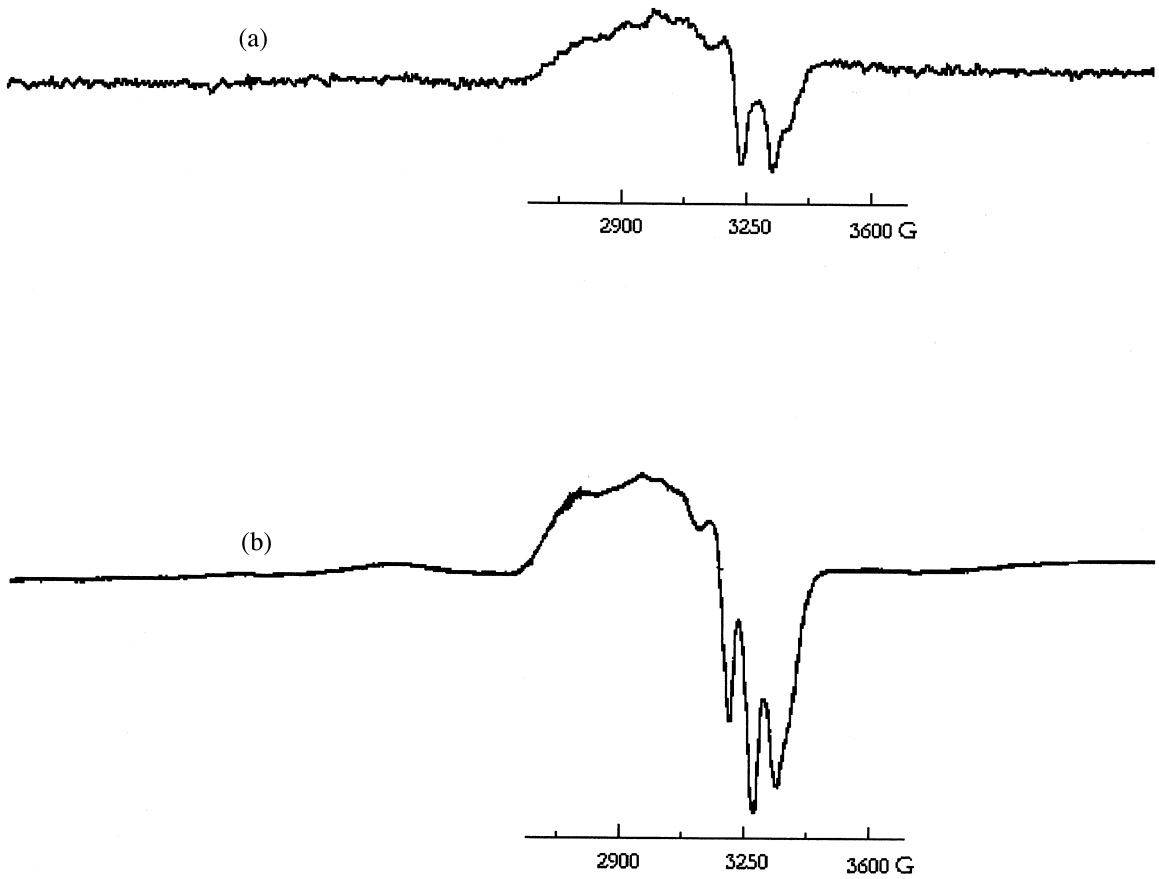

Fig. 6. EPR spectrum of hemin-^{15}NO (a) from the reaction of hemin (2.2 mM) with [^{15}N]hydroxyurea (0.433 M). The $g = 2$ signal corresponds to a field of 3250 G. Trace (b) shows the spectrum of hemin-^{14}NO from the reaction of hemin (2.2 mM) with [^{14}N]hydroxyurea. Hyperfine splitting in hemin-^{15}NO (a) and hemin-^{14}NO (b) was produced by reaction with inositiol hexaphosphate (IHP, 1 mM, Sigma) prior to freezing.

iron oxidized (met-like) products. While these spectra resemble the spectra of the corresponding met-proteins, small differences and the lack of perfect isosbestic points suggest the presence of another product. EPR studies indicate that *X*-NO (X = Mb, HbA, or HbS) forms in these reactions.

The rate constants for the reaction of hydroxyurea with oxyHbA and oxyHbS are similar at both 37°C and room temperature and both reactions appear to be pseudo-first order. The similar rate and product distribution from the reaction of HU with oxyHbA and oxyHbS suggests that the β-6 mutation does not affect the reaction. While the reaction of hydroxyurea and oxyMb produces similar products, the kinetic data shows that this reaction occurs at an increased rate compared with HbA and HbS. As myoglobin does not posses any cysteine residues that correspond to the β-93 cysteines of hemoglobin, this increased rate may indicate the involvement of these residues in these reactions. However, blocking the β-93 cysteines in HbA with iodoacetamide followed by reaction with HU, resulted in no significant differences in rate and product distribution. These results demonstrate that the β-93 cysteines do not influence the reaction of HbA and HbS with hydroxyurea and do not account for the differences in rate of reaction between hemoglobin and myoglobin with hydroxyurea. The increased rate of the oxyMb-HU reaction compared to that with

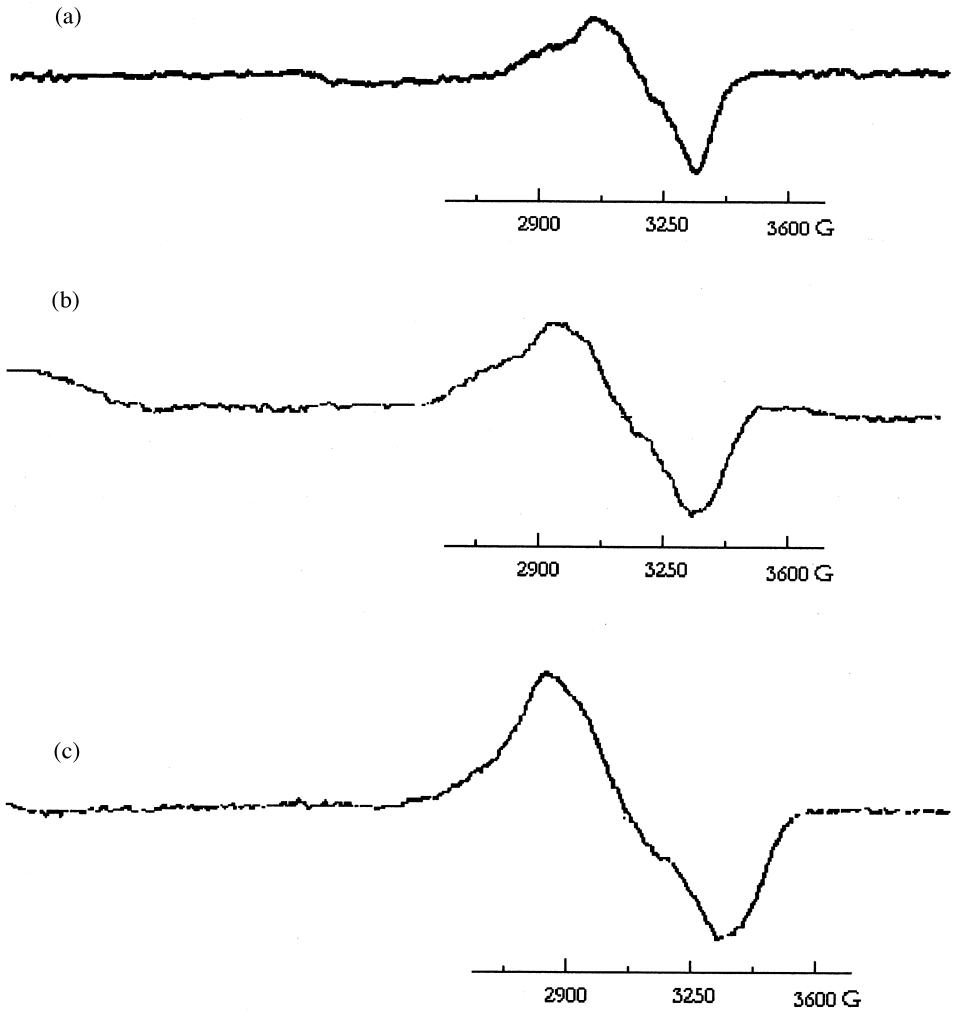

Fig. 7. EPR spectra of metMb (2 mM) and HU (1.32 M) (a), metHbA (1 mM) and HU (1.32 M), and metHbS (1 mM) and HU (1.32 M) (c). The $g = 2$ signal corresponds to a field of 3250 G. The samples were probed at 9.1 GHz with the magnetic field sweeping 1000 G centered at 3250 G.

hemoglobin maybe due to differences in protein structure at the active site. As HU binds heme itself, the structure of the heme pocket of myoglobin may stabilize this binding or be more conducive to some other aspect of the chemistry of the reaction [8,9].

The reaction of hemin with hydroxyurea indicates that the globin portion of the macromolecule is not required for reaction. The rate constants of the hemoglobin proteins do not drastically differ from those of hemin and EPR experiments demonstrate the formation of hemin-NO. As hemin exists in the Fe^{3+} state, this reaction more closely models the reaction of met$X$ (X = Mb, HbA, and HbS) with hydroxyurea rather than the reaction of oxy$X$ with hydroxyurea. Eq. (1) depicts a proposed reaction sequence where the oxygenated forms of Mb, HbA, and HbS react with HU to form met$X$, which reacts further with excess HU to form X-NO. In support of this sequence, metHb has been shown to react with HU to form HbNO [15], and samples of pure

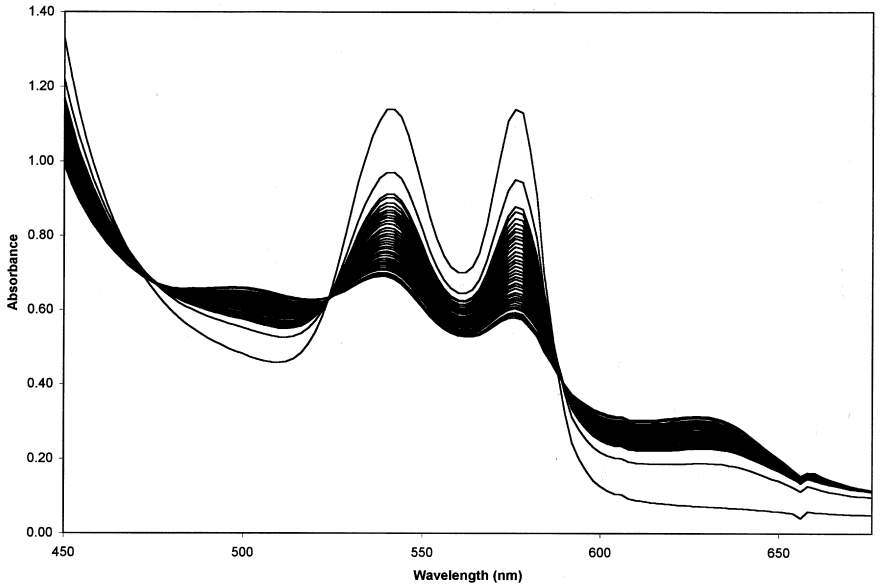

Fig. 8. Time resolved absorption of the reaction of hydroxyurea (660 mM), H_2O_2 (30 mM) and oxyHbA (270 μM) at 37°C. Measurements are shown for 100-s intervals for 1 h. The dip in the spectrum at 655 nm is the result of instrumental error.

met-Mb, met-HbA and met-HbS treated with HU formed MbNO, HbANO, and HbSNO, respectively, as determined by EPR (Fig. 7).

$$X\text{-}O_2 + \mathrm{HU} \rightarrow \mathrm{met}\text{-}X + \mathrm{HU} \rightarrow X\text{-}\mathrm{NO} \qquad (1)$$

If Eq. (1) is true, all *X*-NO formation occurs through the met-protein. This proposed sequence leads to the prediction that *X*-NO formation could be blocked by the addition of a species that binds tightly to Fe^{3+}. Preliminary studies of the reactions of both oxyMb and metMb with hydroxyurea in the presence of KCN show no MbNO formation by EPR (data not shown).

The in vitro experiments reported here demonstrate that the globin portion of these molecules has little influence on the rate or product distribution of the reaction of HU and these proteins. These experiments identify the heme portion of Mb, HbA, HbS as responsible for the oxidation of HU, and the formation of *X*-NO appears to occur through the met species. Previous in vitro experiments demonstrate the formation of NO or 'NO-like' products from the oxidation of HU with hydrogen peroxide or mixtures of hydrogen peroxide and copper(II) sulfate [19]. Indeed, the addition of two equivalents of hydrogen peroxide accelerates the reaction of oxyHbA with HU (Table 2, Fig. 8). These results suggest that the reaction of oxyHb and HU may be especially important in situations of high peroxide concentration and lend support to the idea that NO may be involved in these reactions.

Recent studies provide evidence of HbNO formation after HU treatment in vivo. EPR spectra performed on rats treated with HU confirms nitrosylhemoglobin formation in vivo [20]. Similar experiments reveal the presence of HbS-NO in patients with sickle cell disease receiving HU orally as treatment for sickle cell anemia [21]. Whether in vivo NO production from HU oxidation occurs from the reaction of HU with oxyHb remains unclear as NO production from HU in liver tissues has been described [20]. The in vivo formation of NO from HU oxidation may also arise from other oxidants including peroxidase, lipoxygenase, peroxidase/H_2O_2, copper, zinc-superoxide dismutase, and the tyrosyl radical from ribonucleotide reductase [22–25]. Indeed, preliminary work with cytochrome *c* shows rapid reduction of the protein in reaction with HU. These results suggest that any heme containing

protein may be capable of oxidizing HU with the formation of NO.

5. Conclusion

Taken together, these results indicate that heme-iron complexes react with HU to form the corresponding FeNO species. The structure of the surrounding protein appears to have little influence on these reactions. The formation of FeNO complexes may occur through the reaction of ferric heme proteins with HU. The detection of these FeNO species suggests the intermediacy of NO in these reactions. The reaction of heme proteins with HU with NO production may represent an important metabolic pathway for HU.

Acknowledgements

This work was supported by grants from the American Heart Association (B98423N awarded to DBK-S and 963031N awarded to SBK) and the National Institutes of Health Comprehensive Sickle Cell Center of the Commonwealth of Pennsylvania.

References

[1] S. Charache, M.L. Terrin, R.D. Moore et al., The investigators of the multicenter study of hydroxyurea in sickle cell anemia, effects of hydroxyurea in sickle cell anemia, N. Engl. J. Med. 332 (1995) 1317–1322.
[2] Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia, S. Charache, F.B. Barton, R.D. Moore et al., Hydroxyurea and sickle cell anemia, Medicine 75 (1996) 300–326.
[3] D.L. Rucknagel, R.D. Levere, Sickle Cell Anemia and Other Hemoglobinopathies, Academic Press, New York, 1975.
[4] V.M. Ingram, A specific chemical difference between the globins of normal human and sickle cell anemia haemoglobin, Nature 178 (1956) 792–794.
[5] S.H. Embury, R.P. Hebbel, N. Mohandas, M.H. Steinberg, Sickle Cell Disease, Raven, New York, 1994.
[6] K.R. Bridges, G.D. Barabino, C. Brugnara et al., A multiparameter analysis of sickle erythrocytes in patients undergoing hydroxyurea therapy, Blood 88 (1996) 4701–4710.
[7] S. Charache, Mechanism of action of hydroxyurea in the management of sickle cell anemia in adults, Semin. Hematol. 34 (1997) 15–21.
[8] K. Stolze, H. Nohl, EPR studies on the oxidation of hydroxyurea to paramagnetic compounds by oxyhemoglobin, Biochem. Pharmocol. 40 (1990) 799–802.
[9] D.B. Kim-Shapiro, S.B. King, C.L. Bonifant, S.K. Ballas, Time-resolved absorption study of the reaction of hydroxyurea with sickle cell hemoglobin, Biochem. Biophys. Acta 1380 (1998) 64–74.
[10] Y. Xu, C.D. Mull, C.L. Bonifant et al., Nitrosylation of sickle cell hemoglobin by hydroxyurea, J. Org. Chem. 63 (1998) 6452–6453.
[11] R.J. Pacelli, J. Taira, J.A. Cook, D.A. Wink, M.C. Krishna, Hydroxyurea reacts with heme proteins to generate nitric oxide, Lancet 347 (1996) 906.
[12] A.J. Gow, J.S. Stamler, Reactions between nitric oxide and haemoglobin under physiological conditions, Nature 391 (1998) 169–173.
[13] M.P. Doyle, S.N. Mahapatro, R.D. Broene, J.K. Guy, Oxidation and reduction of hemoproteins by trioxodinitrate(II), J. Am. Chem. Soc. 110 (1988) 593–599.
[14] G.L. Geraci, J. Parkhurst, G.H. Gibson, Preparation and properties of α- and β-chains from human hemoglobin, J. Biol. Chem. 17 (1969) 4664–4667.
[15] D.B. Kim-Shapiro, S.B. King, H. Shields, C.P. Kolibash, W.L. Gravatt, S.K. Ballas, The reaction of deoxy-sickle cell hemoglobin with hydroxyurea, Biochem. Biophys. Acta 1428 (1999) 381–387.
[16] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.
[17] H. Kon, J. Biol. Chem. 243 (1968) 4350–4357.
[18] N.F. Ramsey, Nuclear Moments, Wiley, New York, 1953.
[19] N.S. Kwon, D.J. Stuehr, C.F. Nathan, Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide, J. Exp. Med. 174 (1991) 761–767.
[20] J. Jiang, S.J. Jordan, D.P. Barr, M.R. Gunther, H. Maedea, R.P. Mason, In vivo production of nitric oxide in rats after administration of hydroxyurea, Mol. Pharmacol. 52 (1997) 1081–1086.
[21] R.E. Glover, E.D. Ivy, E.P. Orringer, H. Maeda, R.P. Mason, Detection of nitrosyl hemoglobin in venous blood in the treatment of sickle cell anemia with hydroxyurea, Mol. Pharmacol. 55 (1999) 1006–1010.
[22] G. Lassmann, B. Liermann, ESR studies of structure and kinetics of radicals from hydroxyurea, Free Rad. Biol. Med. 6 (1989) 241–244.
[23] W. Chamulitrat, R.P. Mason, D. Riendeau, Nitroxide metabolites from alkylhydroxylamines and *N*-hydroxyurea derivatives resulting from reductive inhibition of soybean lipoxygenase, J. Biol. Chem. 267 (1992) 9574–9579.
[24] K. Sato, T. Akaike, T. Sawa et al., Nitric oxide generation from hydroxyurea via copper-catalyzed peroxidation and implications for pharmacological actions of hydroxyurea, Jpn. J. Cancer Res. 88 (1997) 1199–1204.
[25] G. Lassman, L. Thelander, A. Graslund, EPR stopped-flow studies of the reaction of the tyrosyl radical of protein R2 from ribonucleotide reductase with hydroxyurea, Biochem. Biophys. Res. Commun. 188 (1992) 879–887.