# Release of Nitric Oxide from *S*-Nitrosohemoglobin. Electron Transfer as a Response to Deoxygenation

John Paul Pezacki,‡ Noam J. Ship, and Ronald Kluger*

*The Davenport Laboratories, Department of Chemistry University of Toronto, 80 St. George St. Toronto, Ontario, Canada M5S 3H6*

*Received February 23, 2001*

Hemoglobin (Hb) is a tetrameric ($\alpha\alpha\beta\beta$) heme protein in circulating red cells that transports and releases oxygen from each of its four ferrous heme groups. The heme irons also bind nitric oxide, a species implicated in the relaxation of blood vessels.[1] Stamler and co-workers found that a small but significant amount of the circulating Hb is nitrosylated at the sulfur of $\beta$-Cys-93 ("HbSNO").[2−8] They observed that a subset of the HbSNO transfers the nitrosyl group to the blood vessel via the anion exchange protein AE1 of the red cell's membrane, causing relaxation.[9] The status of HbSNO is allosterically controlled by transfers of NO to the ferrous heme of hemoglobin (HbNO, eq 1), RSNOs, such as *S*-nitrosoglutathione (GSNO) and *S*-nitrosylated AE1[8] (eq 2). These equilibria regulate blood flow[2−8] and platelet activity.[10]

$$\underset{\text{T state}}{\text{HbNO}} \rightleftharpoons \underset{\text{R state}}{\text{HbSNO}} + e^- + H^+ \quad (1)$$

$$\underset{\text{R state}}{\text{HbSNO}} + \text{RSH} \rightleftharpoons \underset{\text{T state}}{\text{Hb(SH)}} + \text{RSNO} \quad (2)$$

We now report a mechanism by which NO can be released directly from HbSNO in response to deoxygenation.[3] We observe the change in HbSNO through cycles of oxygenation and deoxygenation by its visible absorption, mass, oxygen affinity ($P_{50}$), and cooperativity ($n_{50}$). Significantly, oxidation of the heme irons of Hb-SNO occurs as NO is released during these cycles, while a control sample of native hemoglobin is stable. After the SNO group of HbSNO is converted to the thiol, the resulting spectrum is intermediate between that of oxyHb and that of ferric hemoglobin (metHb), with 50% of the heme iron oxidized. The extent of NO release (measured by ESI MS) coincides with the extent of metHb formation. Samples of HbSNO were carefully prepared as reported previously[5,7a] in the presence of EDTA to remove metal contaminants. The $P_{50}$ and $n_{50}$ also parallel the conversion from HbSNO to metHb. HbSNO binds oxygen more tightly than does native Hb.[7a] Limiting values are $P_{50} = 8$ Torr and $n_{50} = 1.6$ at pH 7.4 at 37 °C while limiting values are $P_{50} =$ 3−4 Torr and $n_{50} = 1.6$ at 25 °C (Figure 1). These are consistent with production of Hb with $Fe^{III}$ in the $\beta$ subunits and $Fe^{II}$ in the $\alpha$-subunits.[11,12]

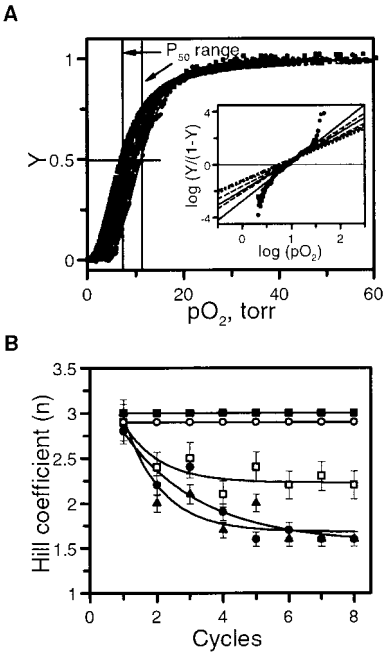



**Figure 1.** (A) Changes in oxygen binding curves and Hill plots (inset) as a function of cycling (37 °C, pH 7.4 phosphate, $\mu$ 0.1 M). **(**B) Changes in $n_{50}$ as a function of cycling for Hb at 37 °C (○), HbSNO at 37 °C (●), HbSNO with 1 SNO/tetramer at 37 °C (□), Hb at 25 °C (■), and HbSNO at 25 °C (▲).

$^1$H NMR analysis of the products reveals that electron transfer occurs from $\beta$-hemes (vs $\alpha$-hemes) derived from HbSNO. We incubated solutions of ~0.1 mM HbSNO under argon for 2.5 and 48 h. The solutions were concentrated and dissolved in deuterium oxide containing a 10-fold excess of sodium azide, which forms iron azides. The 500 MHz $^1$H NMR signals for the $CH_3$ groups of positions 1-, 5-, and 8- of the heme in the $\alpha$ and the $\beta$ subunits are resolved.[13] The methyl protons undergo a paramagnetic shift when the heme is the ferric azido derivative (confirmed in the spectrum of azido metHb (where the all heme iron is Fe(III)). The product that was analyzed after 2.5 h had about 30% of its hemes oxidized: the $\beta$ hemes to a greater extent than $\alpha$ (3−4:1, Figure 2). The sample that was analyzed after 2 days of reaction had about 50% of the total heme oxidized, with the Fe(III) equally distributed among the hemes, consistent with initial oxidation by transfer of an electron to the HbSNO from the $\beta$ heme to produce an adjacent ferric heme. Slow transfer of electrons among the hemes occurs as an epiphenomenon, accounting for the presence of an increasing amount of oxidation of the $\alpha$ hemes. The $\beta$ heme ferrous ions donate electrons to the adjacent S−NO causing reductive cleavage to yield the cysteine thiolate and NO. The $\beta$ sites in tetramers then exchange, distributing oxidation randomly among $\alpha$ and $\beta$ sites.[14]

Although NO may react rapidly with oxyHb (eq 4), under the conditions of our experiments, the nascent NO binds to Fe(II) in a heme or is carried off in the flow of inert gas. Circular dichroism

‡ Present address: The National Research Council of Canada, Steacie Institute for Molecular Sciences, 100 Sussex Dr., Ottawa, Canada K1A 0R6. E-mail: rkluger@chem.utoronto.ca.

(1) Ignarro, L. J. *Hypertension* **1990**, *16*, 477.
(2) Gow, A. J.; Luchsinger, B. P.; Pawloski, J. R.; Singel, D. J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9027.
(3) Gow, A. J.; Stamler, J. S. *Nature* **1998**, *391*, 169.
(4) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J. S. *Nature* **1996**, *380*, 221.
(5) McMahon, T. J.; Stamler, J. S. *Methods Enzymol.* **1999**, *301*, 99.
(6) Stamler, J. S.; Jia, L.; Eu, J. P.; McMahon, T. J.; Demchenko, I. T.; Bonaventura, J.; Gernert, K.; Piantadosi, C. A. *Science* **1997**, *276*, 2034.
(7) (a) Patel, R. P.; Hogg, N.; Spencer, N. Y.; Kalyanaraman, B.; Matalon, S.; Darley-Usmar, V. M. *J. Biol. Chem.* **1999**, *274*, 15487. (b) Wolzt, M.; MacAllister, R. J.; Davis, D.; Feelisch, M.; Moncada, S.; Vallance, P.; Hobbs, A. J. *J. Biol. Chem.* **1999**, *274*, 28983.
(8) McMahon, T. J.; Stone, A. E.; Bonaventura, J.; Singel, D. J.; Stamler, J. S. *J. Biol. Chem.* **2000**, *275*, 16738 and references therein.
(9) (a) Pawloski, J. R.; Hess, D. T.; Stamler, J. S. *Nature* **2001**, *409*, 622. (b) Gross, S. S. *Nature* **2001**, *409*, 577.
(10) Pawloski, J. R.; Swaminathan, R. V.; Stamler, J. S. *Circulation* **1998**, *97*, 263.
(11) Shibayama, N.; Imai, K.; Morimoto, H.; Saigo, S. *Biochemistry* **1993**, *32*, 8792.
(12) Perutz, M. F.; Wilkinson, A. J.; Paoli, M.; Dodson, G. G. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 1.
(13) Yamamoto, Y.; Nagaoka, T. *FEBS Lett.* **1998**, *424*, 169 and references therein.
(14) Shibayama, N.; Morimoto, H.; Saigo, S. *Biochemistry* **1998**, *37*, 6221 and references therein.

10.1021/ja015716o CCC: $20.00 
Published on Web 04/20/2001

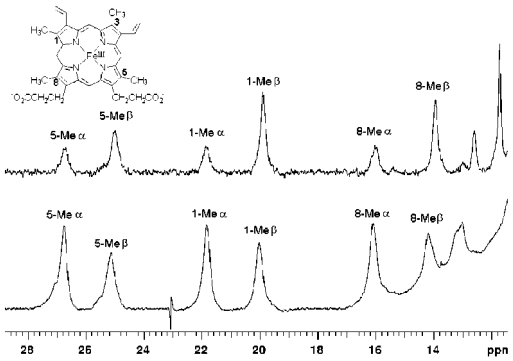



**Figure 2.** $^1$H NMR spectra of the heme portion of HbSNO under argon at room temperature for 2.5 (upper) and 48 h (lower) (oxygenated prior to analysis) in deuterium oxide, collected at 45 °C, ~10-fold excess of azide.

also shows a half-met Hb that retains structure and features of the globin chains of Hb containing Fe(II) and Fe(III).

We observe small amounts (~5−10%) of HbNO during deoxygenation cycles of HbSNO as changes in the soret band ($\lambda$ = 418 nm). As NO escapes from the pocket near Cys-93, it may be dispersed into solution and bind to ferrous hemes.

Related routes for formation of MetHb are heterolytic cleavage of $Fe^{II}NO$, producing Fe(III) and nitroxyl ion ($NO^-$), eq 3,[2] and the reaction of NO with oxyHb, eq 4. [2,15]

$$HbFe^{II}NO + H^+ \rightarrow HbFe^{III} + \frac{1}{2}N_2O + \frac{1}{2}H_2O \qquad (3)$$

$$HbO_2 + {}^{\bullet}NO \rightarrow metHb + NO_3^- \qquad (4)$$

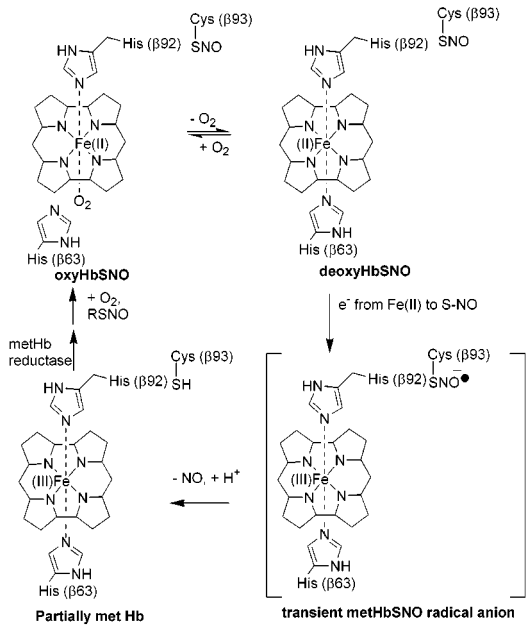
Neither reaction leads to release of NO from $\beta$-Cys93-SNO in deoxygenated solutions, which is the key feature of the reactions in eqs 1, 3, and 4. Furthermore, NO is swept from our system, keeping the concentration very low. MetHb forms from HbSNO in the absence of oxygen ($t_{1/2}$ ~ 26 min), giving NO and $N_2O$. However, *S*-nitrosothiols are good electron acceptors.[16] Thus, transfer of an electron from the $\beta$-heme iron to $\beta$-Cys93-S-NO accounts for triggering of S-NO cleavage by deoxygenation (Scheme 1). The resulting anion radical (or transition state) releases nitric oxide to generate the free Cys93 thiolate, which is rapidly protonated. In the red cell, metHb reductase will convert metHb to the ferrous state (Scheme 1). This chemical mechanism is consistent with oxygen-dependent release of NO from HbSNO (in the absence of endogenous thiols that react via transnitrosation reactions) accompanied by formation of metHb (Scheme 1).

The specific response of NO release from HbSNO to deoxygenation can be rationalized in terms of reduction potentials. The reduction potential for Cu(II)/Cu(I) is 0.15 V; NO is released from RSNO compounds (eq 5) by electron transfer from Cu(I).[11] The potential for $Fe^{III}/Fe^{II}$ within Hb is ~0.17 V.[17]

$$RSNO + Cu^I \rightarrow RS^- + {}^{\bullet}NO + Cu^{II} \qquad (5)$$

Above pH 8.0, oxyHb reacts rapidly with *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acylpenicillamine (SNAP) exclusively at the $\beta$-Cys93.[18,19] However, prolonged exposure leads to metHb formation. We excluded the possibility that nitrite, which can be present in samples of RSNO, is responsible for metHb formation by rigorously purifying the RSNO's. Reactions of deoxyHb with GSNO or SNAP result in rapid heme oxidation ($k_{ox}$ ~ 14.5 $s^{-1}$) and formation of disulfides at $\beta$cys93 (25 °C, pH 7.4). OxyHb reacts more slowly ($k_{ox}$ ~ 5 × $10^{-4}$ $s^{-1}$). The reactions of horse heart deoxymyoglobin (deoxyMb), without cysteines, occur with rate constants similar to those for deoxyHb. The mechanism in Scheme 1 requires protonation of the cysteine thiolate as NO is released. pH-stat measurements of the rate of proton uptake from the HbSNO samples that release NO gave the same result as those measured for heme oxidation.

**Scheme 1**




The $\beta$-Cys93 thiol is 12.9 Å from the closest ferrous heme in deoxyHb,[20] close enough for a direct electron transfer.[21,22] The distance between the centers influences the rates of electron transfer.[23] The ligation state of the ferrous ion will affect its reduction potential. Coordination of oxygen, an electron withdrawing ligand, increases the heme $Fe^{III}/Fe^{II}$ potential, making it a poorer electron donor.[24] Thus, oxygen would be an allosteric effector for NO release, with dissociation permitting internal electron transfer to occur.

In summary, we have found an internal electron-transfer mechanism, triggered by depletion of oxygen, for selective heme-assisted release of NO from HbSNO. This mechanism may relate to the role of HbSNO in NO-based signaling in blood vessels.

**Acknowledgment.** We thank the Natural Sciences and Engineering Research Council of Canada for grant support (R.K.) and a postdoctoral fellowship (J.P.P.).

JA015716O

(15) Eich, R. F.; Li, T.; Lemon, D. D.; Doherty, D. H.; Curry, S. R.; Aitken, J. F.; Mathews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N., Jr.; Olson, J. S. *Biochemistry* **1996**, *35*, 6976.

(16) Arulsamy, N.; Bohle, D. S.; Butt, J. A.; Irvine, G. J.; Jordan, P. A.; Sagan, E. *J. Am. Chem. Soc.* **1999**, *121*, 7115.

(17) Cowan, J. A. In *Inorganic Biochemistry An Introduction*; VCH: New York, 1993; Chapter 5.

(18) Williams, D. L. *Acc. Chem. Res.* **1999**, *32*, 869.

(19) Pezacki, J. P.; Pelling, A.; Kluger, R. *J. Am. Chem. Soc.* **2000**, *122*, 10734.

(20) Chan, N. L.; Rogers, P. H.; Arnone, A. *Biochemistry* **1998**, *37*, 16459.

(21) (a) Gruschus, J. M.; Kuki, A. *J. Phys. Chem. B* **1999**, *103*, 11407. (b) Langen, R.; Chang, I. J.; Germanas, J. P.; Richards, J. H.; Winkler, J. R.; Gray, H. B. *Science* **1995**, *268*, 1733.

(22) (a) Moser, C. C.; Keske, J. M.; Warncke, K.; Farid, R. S.; Dutton, P. L. *Nature* **1992**, *355*, 796. (b) Page, C. C.; Moser, C. C.; Chen, X.; Dutton, P. L. *Nature* **1999**, *402*, 47. (c) Beratan, D. N.; Betts, J. N.; Onuchic, J. N. *Science* **1991**, *252*, 1285.

(23) Local movement of the $\beta$-Cys-93 thiol, bringing it closer to iron, is not restricted to either the R or the T state.

(24) The oxidation of hemoglobin by ferricyanide is analogous, requiring oxygen to dissociate first.