S-Nitrosylation of Cross-Linked Hemoglobins at β-Cysteine-93: Stabilized Hemoglobins as Nitric Oxide Sources

John Paul Pezacki, Andrew Pelling, and Ronald Kluger*

Lash Miller Laboratories
Department of Chemistry, University of Toronto
Toronto, Ontario, Canada M5S 3H6

Received August 8, 2000

The endothelium of a blood vessel contains receptors that respond to the presence of nitric oxide and S-nitrosothiols, inducing relaxation of the smooth muscles that constrict the vessel in the immediate area.[1] The discovery that a small but significant portion of hemoglobin in red blood cells occurs as the S-NO derivative of β-cys93 (HbSNO) suggested that this species has a function in the transfer or release of NO groups to increase local blood flow.[2−8] Scavenging of NO will thus cause local constriction and a corresponding increase in blood pressure. Such activity has been ascribed to cross-linked hemoglobins that are being considered as red cell replacements in transfusions.[9] Cross-linking of hemoglobin is necessary when this molecule is used in circulation outside the red cell, preventing dissociation into dimers (which pass through the kidney) and correcting the inappropriately high oxygen affinity (effectors in the red cell promote dissociation of oxygen).[10−14] The heme irons in cross-linked hemoglobins in circulation can bind either oxygen or nitric oxide, accounting for their scavenging effects. While distinguishing between oxygen and NO might solve this problem, an alternative is to have the cross-linked hemoglobin serve as a source of NO. We have now prepared and characterized the first S-nitrosylated cross-linked hemoglobins and have demonstrated the ability of these materials to release NO at a useful rate. These can function as a *circulating cell-free source of NO*, to counter the vasoconstrictive effects of cross-linked hemoglobins circulating outside the red cell.

We prepared S-nitrosylated hemoglobin **1** and two cross-linked S-nitrosohemoglobins **2** and **3** (Figure 1). Reaction of human hemoglobin A ("HbA") with 1,3,5-trimesoyltris(3,5-dibromosalicylate) (TTDS) followed by purification gave the bis-trimesyl amide derived from the ϵ-amino groups of lys-82 of each of the β subunits ("bis-trimesyl-Hb").[15,16] HbA was also cross-linked as the bis-fumaryl amide derived from the ϵ-amino groups of lys-99 of the α-subunits ("bis-fumaryl-Hb") with bis(dibromosalicyl)-fumarate (DBSF) in the presence of inositol hexaphosphate.[17] The cross-linked hemoglobin tetramers were separated from the reagents through a column of Sephadex G25 and purified by anion-exchange HPLC through a column containing the ion-exchange resin AX 300.[18]

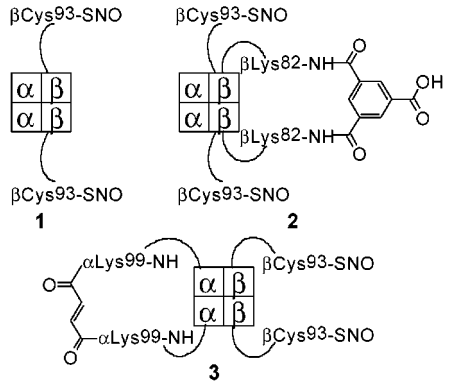


Figure 1. Schematic structures of S-nitrosohemoglobins **1**−**3**.

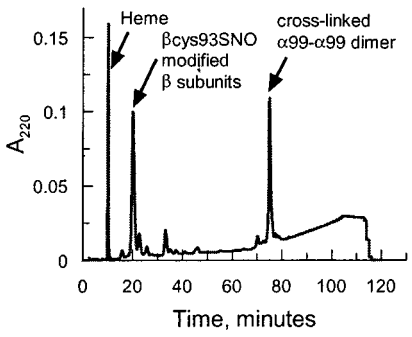


Figure 2. C4 reverse-phase HPLC chromatogram for S-nitrosohemoglobin **3** under dissociating conditions.

HbA, bis-trimesyl-Hb, and bis-fumaryl-Hb, were each converted to S-nitrosyl derivatives by reaction at 4 °C for ∼15−30 min with 0.05 M S-nitroso-*N*-acetylpenicillamine (SNAP)[19] in pH 9.0 borate (0.05 M) containing 0.005 M EDTA. The S-nitrosylated hemoglobins were separated from SNAP using Sephadex G25 columns. Highly pure materials were produced as determined by HPLC methods.[2,18] S-nitrosylation of the βcys93's were confirmed using the Savalle Assay and by ESI mass spectrometry.[2] Purity of the hemoglobin conjugates was evaluated by C4 reverse-phase HPLC (Figure 2).

The NO groups are readily transferred to acceptors. The reaction of **2** or **3** with glutathione (GSH) yields S-nitrosoglutathione (GSNO) (eq 1), confirming that **2** and **3** can donate NO to biological thiols as does native hemoglobin.[3−8] They may also release NO directly in a redox coupled reaction with their internal heme iron.[20]

$$\text{cross-linked HbSNO} + \text{RSH} \rightleftharpoons \text{cross-linked Hb(SH)} + \text{RSNO} \qquad (1)$$

The rates of transfer of the nitrosyl group from SNAP to oxyhemoglobin and modified oxyhemoglobins were measured

(1) Ignarro, L. J. *Hypertension* **1990**, *16*, 477−483.
(2) McMahon, T. J.; Stone, A. E.; Bonaventura, J.; Singel, D. J.; Stamler, J. S. *J. Biol. Chem.* **2000**, *275*, 16738.
(3) 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.
(4) Gow, A. J.; Stamler, J. S. *Nature* **1998**, *391*, 169.
(5) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J. S. *Nature* **1996**, *380*, 221.
(6) McMahon, T. J.; Stamler, J. S. *Methods. Enzymol.* **1999**, *301*, 99.
(7) 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.
(8) (a) Patel, R. P.; Hogg, N.; Spencer, N. Y.; Kalyanaraman, B.; Matalon, S.; Darley-Usmar, V. M. *J. Biol. Chem.* **1999**, *274*, 15487.
(9) Winslow, R. M. *Annu Rev. Med.* **1999**, *50*, 337.
(10) Kluger, R. In *Protein Function A Practical Approach*; Creighton, T. E., Ed.; URL Press: Oxford, U.K., 1997; pp 185−214.
(11) Kluger, R.; Shen, L.; Xiao, H.; Jones, R. T. *J. Am. Chem. Soc.* **1996**, *118*, 8782.
(12) Schumacher, M. A.; Dixon, M. M.; Kluger, R.; Jones, R. T.; Brennan, R. G. *Nature* **1995**, *375*, 84.
(13) Schumacher, M. A.; Zheleznova, E. E.; Poundstone, K. S.; Kluger, R.; Jones, R. T.; Brennan, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 7841.
(14) Fernandez, E. J.; Abad-Zapatero, C.; Olsen, K. W. *J. Mol. Biol.* **2000**, *296*, 1245.
(15) Walder, J. A.; Zaugg, R. H.; Walder, R. Y.; Steele, J. M.; Klotz, I. M. *Biochemistry* **1979**, *18*, 4265.
(16) Kluger, R.; Song, Y.; Wodzinska, J.; Head, C.; Fujita, T. S.; Jones, R. T. *J. Am. Chem. Soc.* **1992**, *114*, 9275.
(17) Walder, R. Y.; Andracki, M. E.; Walder, J. A. *Methods Enzymol.* **1994**, *231*, 274.
(18) Jones, R. T. *Methods Enzymol* **1994**, *231*, 322.
(19) SNAP was prepared by reacting equimolar amounts of sodium nitrite and *N*-acetylpenicillamine in acidic medium. SNAP was purified by column chromatography in the dark to remove unwanted sodium nitrite.

10.1021/ja005516x CCC: $19.00
Published on Web 10/18/2000

Table 1. Rate Constants for NO Transfer (k_{RSNO}) from SNAP to Hemoglobin and Cross-linked Hemoglobin at 25 °C, $I = 0.1$ M (KCl)

hemoglobin	pH	k_{RSNO}, $M^{-1}s^{-1}$	k_{rel}
HbA[a]	7.4	1.20 ± 0.08	1.0
bis-trimesyl-Hb[a]	7.4	$(2.41 \pm 0.10) \times 10^{-1}$	0.2
bis-fumaryl-Hb[a]	7.4	1.42 ± 0.09	1.2
HbA[b]	9.0	$(6.22 \pm 0.05) \times 10^{2}$	1.0
bis-trimesyl-Hb[b]	9.0	$(9.71 \pm 0.09) \times 10^{1}$	0.15
bis-fumaryl-Hb[b]	9.0	$(6.83 \pm 0.06) \times 10^{2}$	1.1

[a] Measured using the metHb form. [b] Measured using the oxyHb form.

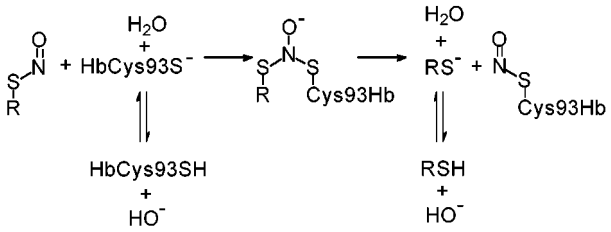
spectrophotometrically with stopped-flow mixing. We follow the disappearance of the signals associated with SNAP (at $\lambda = 336$, or 540). The signal for SNAP was stable in the absence of the hemoglobin substrates. SNAP is a sterically hindered NO donor that can undergo competing oxidation reactions. Despite this, S-nitrosylation of hemoglobin is achieved with limited oxidation of the donor. The values for the second-order rate constants (k_{RSNO}) were determined by plotting the observed rate coefficients as a function of SNAP concentration. For reactions at pH 7.4, oxidation of the ferrous ions of the hemes is a kinetically significant competing reaction.[20] The competitive reaction is avoided if the hemes are oxidized first using ferric cyanide (eq 2). The reagent was removed by centrifugal ultrafiltration prior to the nitrosylation reaction. Thus, the rates of transfer at pH 7.4 were measured using the heme-iron oxidized proteins. Methemoglobin has tertiary and quaternary structures that are similar or isomporphous to oxyhemoglobin, depending on pH and spin state of the Fe's.[21]

$$\text{oxyHb} + \text{Fe(III)(CN)}_6^{3-} \rightarrow \text{metHb} + \text{Fe(II)(CN)}_6^{4-} \quad (2)$$

The second-order rate constants for trans-nitrosation from SNAP to HbA, bis-trimesyl-Hb, and bis-fumaryl-Hb were measured at pH 7.4 and at pH 9.0. Those results are summarized in Table 1.

The pH dependence of transfer of the NO group from SNAP to HbA (to form Hb-SNO), bis-trimesyl-Hb, and bis-fumaryl-Hb indicates that the transfer reactions are faster at higher pH. Williams demonstrated that thiolates are the reactive species in analogous trans-nitrosation reactions.[22] It is likely that the thiolate of the βcys93 side chain reacts with S-nitrosothiols according to Scheme 1.

Scheme 1



Additional contributions to the differences in rates of nitrosyl transfer at neutral and alkaline solutions may result from the disruption of the salt bridges responsible for the alkaline Bohr effect,[21] which also stabilizes the R state. This effect may lower the pK_a of β-cys93-SH as well as give greater exposure to β-cys93.

The rate coefficients for nitrosyl transfer from SNAP to cross-linked hemoglobins indicate that the β-lys82 cross-linked material (bis-trimesyl-Hb) reacts more slowly with S-nitrosothiols relative to the native hemoglobin, whereas the α-lys99 cross-linked hemoglobin (bis-fumaryl-Hb) reacts somewhat faster than does native hemoglobin.

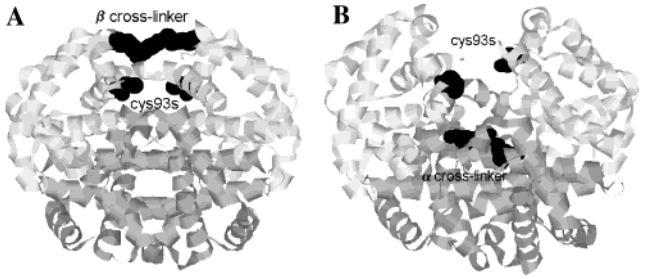


Figure 3. **A** is the model of **2** and **B** is the model of **3** (both R state).

The bioactivity of S-NO hemoglobin within the red cell differs markedly from that of other NO donors in that it is linked to the degree of oxygenation (and quaternary structure). Exposure of the S−H of β-cys93 is increased in the deoxygenated T state of the protein (eq 3).[2,7] Cross-linked S-NO hemoglobins thus form a distinct class of synthetic NO group donors with unique properties.

$$\underset{\text{T state}}{\text{deoxyHbSNO}} + \text{GSH} \rightleftharpoons \text{deoxyHb(SH)} + \text{GSNO} \quad (3)$$

The cross-linked HbSNO's experience restricted motion.[9,11,12] Although they are cooperative and do undergo geometric changes (R to T), these are less complete than in native hemoglobin.[11,12] This suggests that the R to T transition may be less important to the relative reactivity of the cys93's than is the positioning of the cys93's in transient structures such as the proposed R2 structure.[21] Important implications for the mechanism for NO release from HbSNO in the red cell arise from such a premise. If an intermediate state, such as R2, is as reactive as the T state (implied by our data), then NO group release would be more sensitive to changes in partial pressure of oxygen than predicted previously.

We examined the likely structures of the cross-linked S-nitrosohemoglobins based on published X-ray structures.[12−13,23] We see that the β-*cross*-linker hinders the approach to reaction at the βcys93. The β-cys93 side chains are located within globins near the interface between β-subunits and below the DPG binding site (Figure 3). Chemical cross-linking of the β-subunits diminishes the space between the β-subunits relative to native hemoglobin that in turn restricts access to the β-cys93's. The α-*cross*-link is remote from βcys93 and cannot block access. In fact, this linkage compresses the spacing of the α-subunits and increases the space between the β-subunits. This increases accessibility to the β-cleft and to βcys93. On the basis of these findings we conclude that the effect of cross-linking on the dynamics of trans-nitrosation reactions is largely steric in nature.

We have demonstrated that cross-linked S-nitrosohemoglobins can be prepared readily and that they retain the ability to transfer NO groups to and from the reactive β-cys93 side chains. The cross-linked S-nitrosohemoglobins thus can serve as stabilized structural and functional analogues of HbSNO and may enhance the clinical effectiveness of cross-linked hemoglobins as red cell substitutes by serving as NO sources in the absence of red cells.

Acknowledgment. We thank the Natural Sciences and Engineering Council of Canada (NSERC) for funding. J.P.P. thanks NSERC for a postdoctoral fellowship.

JA005516X

(20) Pezacki, J. P.; Kluger, R. Submitted for publication.
(21) Perutz, M. F.; Wilkinson, A. J.; Paoli, M.; Dodson, G. G. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 1.
(22) Williams, D. L. *Acc. Chem. Res.* **1999**, *32*, 869.
(23) Chan, N. L.; Rogers, P. H.; Arnone, A. *Biochemistry* **1998**, *37*, 16459.