Biochemical Characterization of Human S-Nitrosohemoglobin

EFFECTS ON OXYGEN BINDING AND TRANSNITROSATION*

(Rceived for publication, November 19, 1998, and in revised form, March 10, 1999)

S. M.), and Office of Naval Research Grant N00014-97-1-0909 (to S. M.).

The costs of publication of this article were defrayed in part by the American Society for Biochemistry and Molecular Biology, Inc.

The Journal of Biological Chemistry
Printed in U.S.A.

Vol. 274, No. 22, Issue of May 28, pp. 15487–15492, 1999

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S-Nitrosation of cysteine β93 in hemoglobin (S-nitrosohemoglobin (SNO-Hb)) occurs in vivo, and transnitrosation reactions of deoxygenated SNO-Hb are proposed as a mechanism leading to release of NO and control of blood flow. However, little is known of the oxygen binding properties of SNO-Hb or the effects of oxygen on transnitrosation between SNO-Hb and the dominant low molecular weight thiol in the red blood cell, GSH. These data are important as they would provide a biochemical framework to assess the physiological function of SNO-Hb. Our results demonstrate that SNO-Hb has a higher affinity for oxygen than native Hb. This implies that NO transfer from SNO-Hb in vivo would be limited to regions of extremely low oxygen tension if this were to occur from deoxygenated SNO-Hb. Furthermore, the kinetics of the transnitrosation reactions between GSH and SNO-Hb are relatively slow, making transfer of NO+ from SNO-Hb to GSH less likely as a mechanism to elicit vessel relaxation under conditions of low oxygen tension and over the circulatory lifetime of a given red blood cell. These data suggest that the reported oxygen-dependent promotion of S-nitrosation from SNO-Hb involves biochemical mechanisms that are not intrinsic to the Hb molecule.

The main function of Hb is transporting oxygen from the lungs to respiring tissues. Oxygen is released when Hb undergoes a conformational change from the “R” state (i.e. “relaxed” or high oxygen affinity form) to the “T” state (i.e. “tense” or low oxygen affinity form). Numerous allosteric mechanisms are present that regulate oxygen delivery by either increasing or decreasing the oxygen affinity of Hb. These include regulation by pH, carbon dioxide, and inorganic phosphates (1, 2). For example, by binding to the T state, which results in stabilization of this conformer, phosphates such as adenosine triphosphate or 2,3-bisphosphoglycerate decrease the oxygen affinity of whole blood relative to purified Hb. These physiological regulatory mechanisms work in concert to ensure that oxygen delivery satisfies metabolic demand.

Efficient oxygen delivery is also a function of the amount of blood flowing through the arteries and arterioles, which, in turn, is regulated by the action of agents that cause either vasorelaxation or vasoconstriction. Nitric oxide is critical in vascular homeostasis and an important physiological modulator of blood flow due to its ability to cause vessel relaxation (3). Nitric oxide is synthesized in endothelial cells via the oxidation of L-arginine, a reaction catalyzed by the type III nitric-oxide synthase and stimulated by physiological processes such as blood flow over the endothelium (4). Once formed, NO diffuses to the underlying smooth muscle cells, where it binds rapidly to the heme group of the enzyme soluble guanylate cyclase. This activates the enzyme and sets up a sequence of events eventually resulting in relaxation of the vessel (5–9). A controversial aspect of this hypothesis is that NO rapidly reacts with oxy-Hb1 (k ~ 10–10 M–1 s–1) (10), and given the high intra-erythrocytic Hb concentration (5–10 mM heme), it is difficult to understand how any NO would be available to initiate the signaling pathway that results in relaxation of vascular smooth muscle (11).

Recent investigations suggest that, in part, the reaction of NO with the heme group of Hb is restricted by partitioning into the hydrophobic compartments of cell membranes, thus making diffusion of the free radical into the red blood cell the limiting process (12). In addition, several investigators have suggested that, in vivo, NO is transported in the thiol-bound, oxidized, and more stable form of an S-nitrosothiol. These compounds are insensitive to a direct reaction with heme groups and have been detected in vivo as both proteins (e.g. S-nitrosoalbumin) and small thiol-containing amino acids or peptides (e.g. S-nitrosoglutathione) (13–20).

Through the formation of S-nitrosothiols, Stamler and coworkers (13, 14) have recently proposed an intriguing hypothesis by which Hb can regulate blood flow and overcome problems associated with scavenging of NO by heme. A novel derivative of the heme protein, S-nitrosohemoglobin (SNO-Hb), was identified as a potential modulator of blood flow in vivo (13), and further investigations have suggested a role for this protein in regulating fetal blood pressure as well (21). S-Nitrosohemoglobin is a derivative in which NO is covalently bonded to the cysteine residue at position 93 on the β-chain (Cys-β93) (22). The nitrosyl group bonded to this thiol may be reduced to NO, under certain conditions, to induce relaxation of pre-capillary vessels and to inhibit platelet aggregation (13, 14, 23). It was suggested that when Hb is in the R conformational

1 The abbreviations used are: oxy-Hb, oxygenated hemoglobin; deoxy-Hb, deoxygenated hemoglobin; SNO-Hb, S-nitrosohemoglobin; deoxy-SNO-Hb, deoxygenated S-nitrosohemoglobin; metHb, methemoglobin or ferric hemeoglobin; GSNO, S-nitrosoglutathione; SNOC, S-nitrosocysteine; SNAP, S-nitroso-N-acetylpenicillamine; DTPA, diethylenetriaminopentaacetic acid; HPLC, high pressure liquid chromatography.
state, the S-nitroso (SNO) group is oriented toward the interior of the protein and stabilized by shielding from the aqueous phase. However, when Hb undergoes transition to the T conformational state, as has been shown to occur in pre-capillary networks, it was proposed that the SNO moiety becomes exposed to the solvent, thereby allowing transnitrosation to glutathione or other thiols present in the red blood cell. The subsequent formation of S-nitrosoglutathione (GSNO) may then promote vessel relaxation (13, 14). In this way, SNO-Hb can increase blood flow and hence oxygen delivery at sites where it becomes deoxygenated. S-Nitrosohemoglobin offers a novel solution by which NO can modulate blood flow without the problems associated with Hb-dependent scavenging of NO.

This hypothesis requires that NO be released following a decrease in the oxygen concentration and thus in the degree of Hb oxygenation in the pre-arterioles. Although it was demonstrated that ligand-induced allosteric changes in Hb affect S-nitrosation of Cys-β93, the published studies have not addressed the effect on the oxygen affinity of Hb. The level of S-nitrosation in vivo (<0.1% of oxy-Hb being S-nitrosated) is insignificant compared with the total oxy-Hb present in the erythrocyte (13). Therefore, the effects of S-nitrosation on oxygen delivery to tissues are negligible. However, since the hypothesis requires that only the deoxygenated conformer of Hb release NO (13, 14), then the oxygen affinity of SNO-Hb (despite its low concentration) is critical to the role of Hb as an NO carrier. This is an important issue because previous studies on the role of Cys-β93 have shown that blockage of the thiol via alkylation or the formation of mixed disulfides increases the oxygen affinity of Hb (24, 25). Furthermore, the naturally occurring Hb variant Hb Okazaki, where Cys-93 is substituted with an arginine residue, also has an increased oxygen affinity (26). Since the formation of SNO-Hb represents a physiologically relevant Hb derivative in which Cys-β93 is modified, we have examined the effect of S-nitrosation of Hb on its oxygen binding properties.

In addition, it is not clear how the oxygen-sensitive mechanisms underlying conformation-dependent NO release function. It has been proposed that transnitrosation reactions between deoxy-SNO-Hb and GSH to form GSNO, the mediator necessary to elicit the vasorelaxant signal, occur. However, transnitrosation reactions are, in general, relatively slow, with the exact kinetics depending on the physiochemical properties of the specific thiols involved. We have therefore also examined the kinetics and equilibria of transnitrosation reactions between Hb and GSNO and fitted the results to a bimolecular reversible reaction.

These data show that S-nitrosation increases the oxygen affinity of Hb, ensuring that if a Hb deoxygenation-sensitive mechanism of NO release exists, then SNO-Hb would only promote vasorelaxation at sites of very low oxygen concentrations, i.e. at sites where the Hb existed primarily in the deoxygenated state. Furthermore, the kinetics of the transnitrosation reaction between SNO-Hb and GSNO are too slow to explain the vasodilator effects of SNO-Hb. These data are discussed in the context of SNO-Hb as a potential modulator of blood flow.

**EXPERIMENTAL PROCEDURES**

Reagents—All reagents were purchased from Sigma. SNOC and SNAP were synthesized as previously reported (27). Human HbA was purified as described below.

**Preparation of Hemoglobin and S-Nitrosohemoglobin—Human hemoglobin was prepared as described previously (10). S-Nitrosohemoglobin was synthesized by incubating oxy-Hb (200 μM; all stated Hb concentrations are in terms of heme) with SNOC (2 mM) at 20 °C for 20–40 min in 2% borate and 1 mM DTPA. In some experiments, SNAP was used instead of SNOC, in which case the incubation time was 1–2 h. All solutions were protected from light. Hb was separated from SNOC or SNAP by gel filtration using Sephadex G-25 (10 × 1 ml; pre-equilibrated with phosphate-buffered saline containing 1 mM DTPA, pH 7.4). When SNOC was used, ~10–20% of the Hb was oxidized to methemoglobin (metHb). This was attributed to the small, but unavoidable concentration of nitrite remaining in our SNOC preparations. No significant metHb (measured by visible spectroscopy) was formed when SNAP was used. Oxy-Hb concentrations were determined by UV-visible spectroscopy using the extinction coefficient per heme group ε_{577} = 14.6 mM^−1 cm^−1 (10). S-Nitrosation of Hb was quantified by the Saville reaction, with all mercury-dependent nitrite formation being protein-precipitable (13, 27). Differing levels of S-nitrosation were obtained by altering the incubation time between oxy-Hb and SNOC or SNAP. Cys-93 thiois on Hb were measured using either dithionitrobenzoic acid or 2,2-dithionitropryridine (28). Hb samples (5 μM heme) were incubated with dithionitrobenzoic acid or 2,2-dithionitropryridine for 20 min at 20 °C, and absorbance at 412 or 386 nm, respectively, was measured. Reduced thiol concentrations were calculated using ε_{512} = 12 mM^−1 cm^−1 or ε_{412} = 14 mM^−1 cm^−1.

**Oxygen Affinities of Hemoglobin and S-Nitrosohemoglobin—Oxygen binding curves of Hb and SNO-Hb were determined using a tonometer. Hemoglobin samples (30–50 μM heme, 3 ml) were incubated initially with a metHb reductase system (29) at 20 °C to remove metHb formed during synthesis of SNO-Hb. The metHb reductase system was found to have no effect on the oxygen affinities of Hb or on the stability of the SNO group (data not shown). Furthermore, similar results were obtained in the absence when the metHb reductase system was not required, i.e. when SNAP was used to synthesize SNO-Hb. Complete reduction to oxy-Hb occurred within 20 min as determined spectrophotometrically. Hb samples were then deoxygenated until deoxy-Hb was formed (determined spectrophotometrically). Samples were deoxygenated until A_{577 and A_{278} = 0.73–0.74. This value was determined from a spectrum of pure deoxy-Hb synthesized by addition of sodium dithionite to a solution of oxy-Hb. Deoxyhemoglobin was accompanied by a decrease in sample volume to between 1.5 and 2 ml. Aliquots of air (0.5–1 ml) were then injected into the tonometer, and the absorbance spectrum (500–700 nm) was recorded after each addition. Oxygen partial pressures were determined by measuring the atmospheric pressure at the time of the experiment using a digital barometer (Fisher). All oxygen binding curves were measured under identical conditions in 50 mM sodium phosphate buffer, pH 7.4, containing 250 μM DTPA at 20 °C and at a constant partial pressure of carbon dioxide.

**Effect of Allosteric Mediators on the Oxygen Affinities of Hb and SNO-Hb—The effect of temperature was assessed by measuring the oxygen binding curve at either 20 or 37 °C. Once deoxygenated, Hb solutions were incubated at the required temperature in a spectrophotometer for 15 min before addition of air. The modulation of oxygen binding by inorganic phosphates was ascertained by adding inositol hexakisphosphate to a 100-fold molar excess to the tetrameric Hb, prior to measurement of oxygen affinity. The effect of pH was assessed by conducting the experiments in 50 mM sodium phosphate buffer containing 250 μM DTPA at pH 6.5 or 7.4.

**Transnitrosation Reactions between SNO-Hb and GSH—To monitor transnitrosation reactions between GSH and deoxy-SNO-Hb, Hb was first deoxygenated in a tonometer, and then GSH (at a concentration 25 times that of heme) was added anaerobically using a gas-tight syringe. Interactions with a mixture of oxygenated and deoxygenated forms were also evaluated by partially oxygenated SNO-Hb before addition of GSH. In either case, the effect of GSH on the oxygen state of Hb was monitored continuously over 5 min by measuring the absorbance spectrum. In parallel experiments, GSNO formation was determined either immediately or 5 min after GSH addition by adding trichloroacetic acid (20% v/v) to Hb in the tonometer. The solution was then centrifuged to remove precipitated protein, and GSNO was measured in the supernatant by the Saville assay.

The reaction between GSNO and either oxy-Hb or deoxy-Hb was also monitored by measuring the rate of loss of GSNO by HPLC. All experiments were performed in 50 mM phosphate buffer, pH 7.4, containing 100 μM DTPA. GSNO concentrations were determined by HPLC using a reverse-phase Kromasil C 18 column (Alltech Associates, Inc.) and a diode array detector.

**Kinetic Analysis of Second-order Reversible Reactions—**Transnitrosation reactions are second-order reversible reactions (Equation 1).
Oxygen Binding and Transnitrosation Reactions of SNO-Hb

The free or S-nitrosated thiols in native oxy-Hb, oxy-SNO-Hb, or oxy-N-ethylmaleimide-Hb were determined as described under "Experimental Procedures." The data shown are representative of SNO-Hb synthesized with varying degrees of S-nitrosation. In this case, SNO-Hb was synthesized by incubation of oxy-Hb (200 μM) with SNAP (2 mM) in 2% borate buffer and 1 mM DTPA at 20 °C for 30 min. In all cases, the total thiold content was 2/ tetramer. The S-nitrosogroup was measured by the Saville reaction, and reduced thiols using dithionitrobenzoic acid. Reactions were carried out in phosphate-buffered saline with 1 mM DTPA at 20 °C. Values represent means ± S.E. (n = 3).

| Form of Hb | SNO/tetramer | Reduced thiols/tetramer | Total thiols (Cys-β93) per tetramer |
|------------|--------------|-------------------------|-----------------------------------|
| Oxy-Hb     |              |                         |                                   |
| Oxy-SNO-Hb | 0.55 ± 0.14  | 1.45 ± 0.19             | 1.91 ± 0.15                       |
| Oxy-NEM-Hb |              |                         | 1.91 ± 0.15                       |

* NEM, N-ethylmaleimide; ND, Not detectable.

The concentration of oxy-Hb for a given pO2 was calculated from the spectra shown in Fig. 1, and the oxygen binding curves for oxy-Hb and SNO-Hb were thus calculated and are shown in Fig. 2A. Native Hb displayed a characteristic sigmoidal oxygen binding curve. S-Nitrosohemoglobin (0.98 SNO groups/tetramer) also had a sigmoidal oxygen binding curve, which was, however, left shifted compared with native Hb (Fig. 2A). These data show that, relative to native Hb, SNO-Hb has a higher oxygen affinity and therefore, under physiological conditions, requires an environment of substantially lower oxygen concentration before releasing oxygen via a deoxygenation-sensitive mechanism as described previously (13, 14). Fig. 2B summarizes a series of experiments in which the oxygen affinities of Hb and SNO-Hb, with differing levels of S-nitrosation, were determined. Oxygen affinities were assessed by comparing the p50 values, i.e. the oxygen tensions at which 50% of the Hb was bound with oxygen. Unmodified Hb had a p50 of 7 ± 0.12 mm Hg (mean ± S.E., n = 12). In each case, S-nitrosation of Hb significantly increased the oxygen affinity (indicated by a decrease in the p50), with the p50 of SNO-Hb (0.6 SNO group/tetramer, i.e. 30% of β93 thiol being S-nitrosated (this number refers to a distribution of SNO groups, as it is not possible to distinguish between specific populations of Hb containing 1 or 2 SNO groups/tetramer) being 4.3 ± 0.27 mm Hg (mean ± S.E., n = 5). Interestingly, S-nitrosation of up to one of the Cys-β93 residues proportionately increased the oxygen affinity; however, further modification did not affect the oxygen affinity (Fig. 2B).

Previous studies found that the SNO group of SNO-Hb was less stable when Hb was in the deoxygenated state, resulting in nitrrosyl-Hb formation, and that this was related to the ability of deoxy-SNO-Hb to promote vasorelaxation (13). Since the method used to determine the oxygen affinity involved initial deoxygenation followed by oxygenation, formation of nitrrosyl-Hb would affect the results obtained. However, no spectral evidence of nitrrosyl-Hb or meth-Hb was seen during the deoxy-endoxy-endoxylation cycle. Furthermore, the concentration of S-nitrosation was measured before and after completion of the oxygen saturation curve. Over the time taken to deoxygenate and then to reoxygenate the Hb (~20–30 min), no
Oxygen Binding and Transnitrosation Reactions of SNO-Hb

The oxygen affinities of Hb and SNO-Hb (0.6–2 SNO groups/tetramer) were measured in the presence and absence of allosteric modulators. The change in oxygen affinity induced by the different allosteric regulators was calculated by measuring the difference in log $p_{50}$. These were then compared to assess the regulatory effects on SNO-Hb relative to Hb. Values represent means ($n = 2$) ± S.E. ($n = 3$, as indicated).

| Allosteric effect | $\Delta \log p_{50}$ (SNO-Hb) | $\Delta \log p_{50}$ (Hb) |
|-------------------|-----------------|-----------------|
| Temperature (37 to 20 °C) | 0.23 | 0.23 |
| IHP* | 0.81 | 0.82 |
| pH 6.5–7.4 | 0.33 ± 0.02 | 0.317 ± 0.05 |
| | 0.96 ± 0.07 |

* IHP, inositol hexaphosphate.

**Table II**

Allosteric modulators have similar effects on the oxygen affinities of Hb and SNO-Hb.

**Fig. 2.** S-Nitration of Hb increases the oxygen affinity. Oxygen binding curves of Hb (●) and SNO-Hb (0.98 SNO group/tetramer; ○) are shown in A and were obtained from the data shown in Fig. 1. Fractional saturation ($Y$) represents [oxy-Hb]/[total Hb]. B shows the effect of S-nitration of Hb on the $p_{50}$ value for oxygen binding. $p_{50}$ values were determined from the respective oxygen binding curves and plotted against the degree of S-nitration of Hb. The data shown were obtained from up to 20 separate experiments and with human Hb obtained from three different donors.

The increased oxygen affinity of SNO-Hb suggests that it is less likely to become deoxygenated and hence release NO under physiological conditions. We have shown that spontaneous release of NO from SNO-Hb is unlikely to be a physiologically relevant mechanism, where a given Hb molecule will be cycling between the oxygenated and deoxygenated forms on a time scale in the order of seconds.

**Effect of Allosteric Mediators on the Oxygen Affinity of SNO-Hb**—The increased oxygen affinity of SNO-Hb suggests that it is less likely than native Hb to become deoxy- and hence release NO under physiological conditions. However, in vivo, numerous regulatory mechanisms modulate the oxygen binding affinity of Hb in the red blood cell. It is possible that S-nitration of Hb alters the sensitivity to the various allosteric regulatory mechanisms present such that SNO-Hb may be able to deliver oxygen under physiological conditions. We therefore tested the effects of pH, temperature, and inorganic phosphates on the oxygen affinity of SNO-Hb and compared it with that of native Hb.

The effects on oxygen affinity of each of the allosteric modulators are shown in Table II and were determined by measuring the difference in log $p_{50}$ of SNO-Hb and Hb elicited by the different allosteric mediators. An increase in temperature is known to decrease the oxygen affinity and thus facilitate oxygen delivery. Table II shows that the change in log $p_{50}$ of Hb and SNO-Hb on increasing the temperature from 20 to 37 °C was the same. Inorganic phosphates decrease the oxygen affinity of Hb (1, 2). Inositol hexaphosphate induced similar increases in the $p_{50}$ of Hb and SNO-Hb, indicating that S-nitrosation of Cys-$\beta$93 does not affect binding of inorganic phosphates to Hb. Finally, we assessed the effect of pH (also called the Bohr effect). The relatively acidic conditions in respiring tissues lower the $p_{50}$ of oxygen binding by Hb, with the net result being oxygen delivery. This occurs in part through binding of protons to histidine residues present in both the $\alpha$- and $\beta$-chains, a property that also serves to buffer plasma. Decreasing the pH from 7.4 to 6.5 decreased the oxygen affinities of both Hb and SNO-Hb to similar extents, with the effect being approximately equal for both forms of Hb (Table II). This shows that S-nitrosation does not significantly affect the binding and release of protons by Hb. It has been calculated that binding of protons by His-$\beta$146 accounts for ~40% of the alkaline Bohr effect (30). The fact that a change in pH exerts similar effects on the oxygen affinities of SNO-Hb and Hb indicates that S-nitrosation of Hb does not significantly alter the biological properties of this histidine residue, which is closely situated to Cys-$\beta$93 (14). Thus, SNO-Hb and Hb are modulated to similar extents by allosteric effector mechanisms.

**Cooperative Nature of Oxygen Binding to SNO-Hb**—A feature of oxygen binding by Hb is that it is cooperative in nature. The effect of S-nitrosation on this property was calculated by Hill plot analysis of the oxygen binding data. Fig. 3 compares the Hill plots for Hb and SNO-Hb. The Hill coefficient at the 50% oxygen saturation level for each Hb was calculated. S-Nitrosation (0.6 SNO group/tetramer) did not significantly decrease the Hill coefficient (2.83 ± 0.11 compared with 3.17 ± 0.11 for unmodified Hb).

We can conclude from the minimal effects of S-nitrosation on the Hill coefficient that the observed changes in oxygen affinity do not involve altered heme interactions. Previous studies have shown that Cys-$\beta$93 is located close to the $\alpha_{1}\beta_{2}$-interface and that its covalent modification inhibits the formation of essential salt links between His-$\beta$146 and Asp-$\beta$94 and of various hydrogen bonds (14). The net effect is a destabilization of the T state and hence an increase in oxygen affinity as well as a decrease in the Hill coefficient. Since S-nitrosation of Hb does not alter the Hill coefficient, the increase in oxygen affinity does not appear to be modulated by affecting the stability of salt links.

**Effect of Glutathione on Release of NO from SNO-Hb**—It has been suggested that transnitrosation reactions between SNO-Hb and low molecular weight thiols, such as GSH, mediate the transfer of the vasorelaxant signal from Hb to the vasculature (13, 14). The mechanism proposed involves transnitrosation from deoxy-SNO-Hb to GSH, with reaction with the $\gamma$ derivative being too slow and thus negligible (13). To examine this point further, GSH was added to a solution of SNO-Hb (2 SNO groups/tetramer) that was partially oxygenated (~60%, i.e. $Y = 0.6$), and the value of $Y$ was measured over 5 min of incubation. Since unmodified Hb has a lower oxygen...
affinity with SNO-Hb (Fig. 2), it is predicted that transnitrosation from SNO-Hb to GSNO, forming Hb and GSNO, will decrease the value of Y. Addition of GSNO heme/GSNO molar ratio ≈ 1:25) to SNO-Hb did not significantly change the value of Y. Addition of GSNO (0.05, 0.01, 0.02, and 0.11 M) to the model and precludes a simple first-order process. The average rate constants for this reaction were 0.13 ± 0.02 M s⁻¹ (kᵣ) and 0.1 ± 0.01 M s⁻¹ (kᵢ). This analysis gives an equilibrium constant for the reaction of 1.3, close to unity. This means that under equilibrium conditions and at the approximately equal concentrations of GSNO and Hb that occur in the red blood cell, the nitroso functional group will be evenly distributed between Hb and GSNO.

The kinetics of transnitrosation under anaerobic conditions (i.e. between deoxy-Hb and GSNO) were also determined as shown in Fig. 4B. The decay of GSNO conformed well to second-order kinetics at high concentrations of GSNO and hemoglobin, but less well at low concentrations. In addition, the rate constants derived from the fits varied dramatically as a function of GSNO concentration (between ~0.01 and 0.1 M s⁻¹ for kᵢ). This suggests that a simple second-order reversible process cannot explain the reaction between GSNO and deoxy-Hb. One mitigating factor precluding accurate kinetic analysis of transnitrosation between GSNO and deoxy-Hb is the observation that GSNO binds to the heme group of deoxy-Hb. Consequently, the amount of GSNO available for transnitrosation will be modulated by the equilibrium of binding to the heme.

Accurate kinetic analysis can only be performed if all reactions are taken into account, and we are currently pursuing this objective. The rate constants for the forward reactions (kᵣ) for both oxy-Hb and deoxy-Hb are slower, but of a similar magnitude to those previously reported (35).

To determine the rate of GSNO formation from SNO-Hb under physiological conditions, simulation of this process was undertaken. Using the rate constants detailed above, the formation of GSNO, under aerobic conditions, from the reaction between 5 μM SNO-Hb and 5 mM GSNO was calculated to be ~3 nM s⁻¹. These concentrations were chosen to approximate the conditions within an erythrocyte. These data indicate that GSNO formation from SNO-Hb is a process capable of slowly generating physiologically relevant concentrations of GSNO (20–100 nM). Although the reactions of deoxy-Hb are kinetically more complex, our data indicate that under anaerobic conditions, the formation of GSNO will be slower than under aerobic conditions.

**DISCUSSION**

The diffusability of NO and the high concentrations of Hb in the circulation indicate that reaction between these two species will occur in vivo. In support of this, the nitrosyl-Hb, a direct product of the reaction between deoxy-Hb and NO, has been
Oxygen Binding and Transnitrosation Reactions of SNO-Hb

detected in the venous circulation (31). The corresponding reaction with oxy-Hb results in formation of metHb and nitrate (32). Since metHb is reduced back to ferrous Hb by the metHb reductase system in erythrocytes and nitrate is an inert end product of NO metabolism, this reaction has been suggested to play a role in the safe removal of NO. However, this reaction also poses a problem regarding how NO, in the presence of Hb, can elicit vasorelaxation and other effects synonymous with this free radical.

The detection of the novel Hb derivative SNO-Hb in vivo and the subsequent description of mechanisms by which this protein can mediate blood flow via NO release have brought new insights into this paradox. This impacts upon the emerging area of NO biology addressing the role of S-nitrosation reactions (i.e. addition of NO) as opposed to nitrosylation (i.e. addition of NO). It was suggested that, in the presence of GSH, the stability of the SNO group of SNO-Hb was dictated by the addition of NO. It was suggested that, in the presence of GSH, the biochemical mechanism that accounts for this observation does not reside within the Hb molecule. An alternative explanation is that the effects on the NO-Hb formation are oxygen-dependent and therefore change throughout the circulation. Indeed, NO-Hb synthesis has been reported to be an oxygen-dependent process (35), and this and our findings that SNO-Hb will be constantly destroyed by GSH indicate that concentrations of this Hb derivative will be higher in the arterial circulation.

Acknowledgments—We thank J. S. Beckman, B. A. Freeman, and M. T. Wilson for helpful discussions.

REFERENCES

1. Kilmarin, J. V. (1976) Br. Med. Bull. 32, 209–222
2. Benesch, R., and Benesch, R. E. (1969) Nature 221, 618–622
3. Palmer, R. M. J., Perreidis, T., and Mencaca, S. (1987) Nature 327, 524–526
4. Kulchan, M. J., Jo, H., and Frangos J. A. (1994) Am. J. Physiol. 267, C753–C758
5. Ignaro, L. J., Degnan, J. N., Baricos, W. H., Kadowitz, P. J., and Wolin M. S. (1982) Biochim. Biophys. Acta 718, 49–59
6. Tranoy, T. G., and Sharma, V. S. (1992) Biochemistry 31, 2847–2849
7. Lincoln, T. M., and Cornwell, T. L. (1993) FASEB J. 7, 328–338
8. Butt, E., Geiger, J., Jarchau, T., Lohmann, S. M., and Walter, U. (1993) Neurochem. Res. 18, 27–42
9. Francis, S. H., Nollett, B. D., Todd, B. W., Wells, J. N., and Corbin, J. D. (1988) Mol. Pharmacol. 34, 5517
10. Antounini, E., and Brunori, M. (1971) Haemoglobin and Myoglobin in Their Reaction with Ligands, North-Holland Publishing Co., Amsterdam
11. Lancaster, J. R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 8137–8141
12. Liu, X., Miller, M. J., Joshi, M. S., Sadowska-Krowicka, H., Clark, D. A. and Lancaster, J. R., Jr. (1998) J. Biol. Chem. 273, 18709–18713
13. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J. S. (1996) Nature 380, 221–226
14. Stamler, J. S., Jia, L., Lu, E. P., McMahon, T. J., Denchenko, I. T., Bonaven- turia, J., Gernert, K., and Pantadosi, C. A. (1997) Science 276, 2034–2037
15. Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Localio, J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 444–448
16. Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., Chen, D., and Stamler, J. S. (1993) Nature 364, 626–632
17. Gaston, B., Reilly, J., Dranen, J. M., Fackler, J., Ramdey, P., Aruelle, D., Mullins, M. E., Sugarbaker, D. J., Cee, C., Singel, D. J., and Stamler, J. S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10957–10961
18. Scharfstein, J. S., Keaney, J. F. J., Stroka, A., Welch, G. N., Vila, J. A., Stamler, J. S., and Localio, J. (1994) J. Clin. Invest. 94, 1432–1439
19. Xu, L., Lu, J. P., Meissner, G., and Stamler, J. S. (1998) Science 279, 234–238
20. Kluge, I., Gutueck-Amalor, U., Zollinger, M., and De, K. G. (1997) J. Neurochem. 69, 2599–2607
21. Funai, R. F., Davidson, A., Seligman, S. P., and Finlay, T. H. (1997) Biochem. Biophys. Res. Commun. 239, 875–877
22. Ferranti, P., Malintri, A., Mamone, G., Sanno, N., and Marino, G. (1997) FEMS Lett. 400, 19–24
23. Pawloski, J. R., Swaminathan, R. V., and Stamler, J. S. (1998) Circulation 97, 263–267
24. Riggs, A. (1961) J. Biol. Chem. 236, 1948–1954
25. Craescu, C. T., Poyart, C., Schaeffer, C., Garrel, M. C., Kister, J., and Beuzard, Y. (1996) J. Biol. Chem. 271, 14710–14716
26. Harano, K., Harano, T., Shihata, S., Ueda, S., Mori, H., and Seki, M. (1984) FEMS Lett. 173, 45–47
27. Faehlist, M., and Stamler, J. S. (eds) (1996) Methods in Nitric Oxide Research, John Wiley & Sons, Inc., New York
28. Winterbourne, C. C. (1990) Methods Enzymol. 186, 265–272
29. Hayashi, A., Suzuki, T., and Shin, M. (1973) Biochem. Biophys. Acta 310, 321–326
30. Kilmarin, J. V., Breen, J. J., Roberts, G. C., and Ho, C. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1246–1249
31. Kosaka, H., and Seiyama, A. (1996) Biochem. Biophys. Res. Commun. 238, 749–752
32. Doyle, M. P., and Hockstra, J. W. (1981) J. Inorg. Biochem. 14, 351–358
33. Tsaï, A. G., Friesenecker, B., Mazzoni, M. C., Kerger, H., Buerk, D. G., Johnson, P. P., and Intaglieta, M. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6590–6595
34. Rossi, R., Lusini, L., Giannerini, F., Lunagarella, G., and Di Simplicio, P. (1997) Anal. Biochem. 254, 215–220
35. Gow, A. J., and Stamler, J. S. (1998) Nature 391, 169–173