Functional Coupling of Oxygen Binding and Vasoactivity in S-Nitrosohemoglobin*

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S-Nitrosohemoglobin (SNO-Hb) is a vasodilator whose activity is allosterically modulated by oxygen (“thermodynamic linkage”). Blood vessel contractions are favored in the oxygenated structure, and vasorelaxant activity is “linked” to deoxygenation, as illustrated herein. We further show that transnitrosation reactions between SNO-Hb and ambient thiols transduce the NO-related bioactivity, whereas NO itself is inactive. One remaining problem is that the amounts of SNO-Hb present in vivo are so large as to be incompatible with life were all the S-nitrosothiols transformed into bioactive equivalents during each arterial-venous cycle. Experiments were therefore undertaken to address how SNO-Hb conserves its NO-related activity; Our studies show that 1) increased O₂ affinity of SNO-Hb (which otherwise retains allosteric responsivity) restricts the hypoxia-induced allosteric transition that exchanges NO groups with ambient thiols for vasorelaxation; 2) some NO groups released from Cys¹⁰³ upon transition to T structure are autocaptured by the hemes, even in the presence of glutathione; and 3) an O₂-dependent equilibrium between SNO-Hb and iron nitrosylhemoglobin acts to conserve NO. Thus, by sequestering a significant fraction of NO liberated upon transition to T structure, Hb can conserve NO groups that would otherwise be released in an untimely or deleterious manner.

The NO group can bind to the hemes and cysteine residues of hemoglobins of microbial, invertebrate, and mammalian origin, forming alternatively Hb(Fe(II))NO³ and SNO-Hb, respectively (1–4). Iron nitrosylhemoglobin and SNO-Hb are in a dynamic, redox-dependent equilibrium (1–4) (Equation 1).

\[
\text{HS-Hb(Fe(II))NO(Fe(II)}_3 + 4O_2 \rightleftharpoons \text{HS-Hb(Fe(II))O}_2 + \text{GSNO} \quad (\text{Eq. 1})
\]

In mammalian hemoglobin, the position of this equilibrium is linked to the protein conformation: SNO-Hb formation is favored in the R (oxy, low spin) structure, and iron nitrosylhemoglobin is formed preferentially in the T (deoxy, high spin) structure (1, 2, 5–9). Conversely, NO group release from thiols of Hb is promoted by deoxygenation and by heme oxidation (T structure, high spin) (1, 5, 10), in keeping with thermodynamic linkage relationships (11–14). SNO-Hb is also in equilibrium with low mass SNOs (1) such as GSNO (Equation 2). High concentrations of GSH thus shift the equilibrium in favor of the deoxy structure (1, 7); where Equation 4 is the effective sum of Equations 2 and 3.

\[
\text{SNO-Hb(Fe(II))O}_2 + GSH \rightleftharpoons \text{HS-Hb(Fe(II))O}_2 + \text{GSNO} \quad (\text{Eq. 2})
\]

These conclusions as well as their physiological relevance are strongly supported by a series of recent findings. 1) SNO-Hb and Hb(Fe(II))NO have been detected in arterial and venous blood of 41 rats (1, 5), 19 human fetuses in utero (15), and 7 patients with sickle cell disease (16). The measurements show that the position of NO binding in hemoglobin is dependent on both pO₂ and location within the circulation. In particular, NO is redistributed among the hemes and thiols of hemoglobin as it transits the lung, placenta, and peripheral vascular bed. 2) An umbilical arterial-venous difference in the amount of low mass SNOs was detected in term infants, in keeping with an equilibrium between GSNO and SNO-Hb (17). 3) Kinetic, mass spectroscopic, and crystallographic data obtained in multiple laboratories (1, 16, 18–23) have unequivocally identified Cys¹⁰³ as a site of NO binding to hemoglobin.

We have further suggested that the equilibria between SNO-Hb and iron nitrosylhemoglobin, on the one hand (Equation 1), and GSNO, on the other hand (Equation 4), have important functional implications for the regulation of blood flow and platelet activity (5, 10). In support of this contention, we have shown both in vitro and in vivo that SNO-Hb dilates blood vessels (1, 5) and inhibits platelets (10) under conditions that promote its T structure, but not under conditions that promote its R structure. Moreover, we have shown that SNO-Hb bioactivity is promoted by low pO₂, thiols, and heme oxidation (1, 5, 10). The vasodilator and antiplatelet activities of SNO-Hb and promoting role of exogenous thiols have recently been confirmed by others (23, 24). We have also demonstrated that SNO-Hb can sense the physiological O₂ gradient in resistance vessels (small arterioles that control blood flow), thereby coupling the release of vasodilator SNO with regional
metabolic needs of the tissue (5). Additional work that supports the importance of SNO-Hb includes the myriad studies of the systemic effects of inhaled NO (25–29), which raises the levels of endogenous SNO-Hb (16). It is tempting to suggest, moreover, that the hypoxia-increased tissue levels of NO in kidneys of animals that had been treated with NO synthesize inhibitors (30) implicate SNO-Hb as the source of the elevated NO.

The levels of SNO-Hb in arterial blood are ~1 μM. Release of such an amount of SNO (from Hb) during each arterial-venous cycle would not, however, be compatible with life or NO biology for two reasons. First, it would cause life-threatening hypotension and shunting of blood. This effect can be understood by appreciating that regulation of blood flow, which is a function of arteriolar diameter to the fourth power (31), requires only low nanomolar SNO (1, 5, 32–34). Second, it would impose an insurmountable metabolic burden on an organism that produces less NO, in toto (35), than the amount that would be turned over by Hb alone (6). Only a small fraction (0.1–1%) of SNO bound to hemoglobin is involved in signaling during arterial-venous transit to regulate blood flow (1, 5). The NO unit is thus quantitatively and functionally dissimilar from O2. Whereas millimolar O2 is delivered by Hb to meet basal metabolic requirements, NO/SNO functions as a signal that is dispensed at nanomolar concentrations. The fundamental challenge to our understanding of SNO-Hb function is therefore not whether enough NO can be released to dilate blood vessels, as recently questioned by some (23, 36, 37), but rather how red blood cells are able to conserve the vast majority of SNO when their Hb is routinely exposed to hypoxic tissue and to ambient intracellular glutathione. We have previously suggested that part of the solution lies in the autacapture by hemes of the NO released from the thiols in Hb (1, 2, 6), i.e. Hb conserves its NO.

In this study, we report on the functional behavior of (SNO)2-Hb(Fe(II)O)2 (here called SNO-Hb) and on the allosteric mechanism that regulate NO group release from this molecule. We show that 1) NO group release from SNO-Hb is favored in the T structure, thus conforming to principles of thermodynamic linkage; 2) as with other S-nitrosothiols, vasorelaxation by SNO-Hb is not mediated by free NO (as recently misapprehended (23)); and 3) by stabilizing thiol-bound NO in the R structure and autacapturing a significant fraction of NO released upon transition to T structure, Hb can conserve NO that would otherwise be released in an untimely, nonspecific, deleterious, or prodriatal manner.

**EXPERIMENTAL PROCEDURES**

**Reagents and Hemoglobin Solutions—**Chemical reagents were purchased from Sigma, except where otherwise stated. Human hemoglobin (HbA2; Apex Bioscience, NC) was purified (>99%) from outdated human blood as described previously (1, 7). The buffer used in the final chromatographic purification process was lactated Ringer’s solution, pH 7.40, containing 0.5 mM EDTA. S-Nitrosohemoglobin—Hb was dissolved against 2% aerated borate, pH 9.20, containing 0.5 mM EDTA. S-Nitrosocysteine (0.5 mM) was prepared immediately before use by reacting 1 mM NaNO2 in H2O with 1 mM L-Cys hydrochloride dissolved in 0.5 N HCl and 0.05 mM EDTA. S-Nitrosocysteine was partially neutralized prior to addition to the hemoglobin solution by dilution in PBS, pH 8.0, containing 0.5 mM EDTA (7). Hb was S-nitrosylated by incubation at room temperature with 10-fold molar excess S-nitrosocysteine; the extent of S-nitrosylation was determined by the duration of incubation. The reaction was stopped by rapid transfer of the mixture to a Sephadex G-25 column equilibrated with PBS, pH 7.40, containing 0.5 mM EDTA. Human HbA2 contains three pairs of cysteine residues: Cys52, Cys102, and Cys108. Mass spectroscopic analysis of the product formed under study conditions revealed that only Cys108 is nitrosylated (data not shown) (16, 19). SNO-Hb samples were made fresh on the day of an experiment, kept on ice, and protected from light. The SNO yield was determined by the Saville method in aliquots from SNO-Hb solutions used for functional studies (1). No data is expressed as a ratio normalized to the spectrophotometrically determined tetrameric Hb concentration. Tetrmeric Hb/SNO-Hb concentration was adjusted to 15 μM final concentration in PBS, pH 7.40 except where otherwise stated. Some binding study results were confirmed at 50 μM in order to check for effects of dimer/tetramer equilibrium (38, 39).

**Equilibrium O2-binding Studies—**SNO-Hb or Hb(Fe(II)O2 was titrated as described above to 15 or 50 μM final tetrameric concentration and added to a sealed glass tonometer fitted with a cuvette. A base-line spectrum at pH 7.40 and with a spectrophotometric scan in the oxygenated state was obtained (Perkin-Elmer, Beaconsfield, United Kingdom). Rigorous deoxygenation was accomplished through several cycles of alternate flushing with ultra-high purity argon gas and applying a measured vacuum. The tonometer was mechanically rotated for 7 min in a 20 °C water bath, and a spectrum was obtained. A second round of deoxygenation was performed to verify the completeness of conversion to deoxy-Hb and its stability. An airtight syringe (Hamilton, Ontario, Canada) was then used to incrementally deliver measured volumes of room air into the tonometer through a rubber septum. Following each injection, the tonometer was rotated in a temperature-controlled (20 °C) water bath for 7 min to allow equilibration between the head space (~300 ml) and the Hb solution (3 ml), and then a visible spectrum was obtained. Injections of air were repeated until a fully oxygenated spectrum was observed.

The tonometer pO2 resulting with each injection was calculated, taking into account the cumulative volume injected, the tonometer volume, the measured barometric pressure, the ambient room temperature, the water bath (tonometer) temperature, and the relative humidity. The fractional O2 saturation at each step was calculated based on a mean of the change in absorbance at each of three characteristic wavelengths (542, 555, and 577 nm) relative to the respective absorbances of the fully oxygenated and fully deoxygenated species studied in the first two and the last steps of the experiment. Fractional O2 saturation (Y) or log[1/(1 – Y)] (Hill plot) was plotted as a function of the logarithm of pO2, except where otherwise stated.

**Analysis of MetHb and Hb(Fe(II))NO Content—**Spectral decomposition procedures using extinction coefficient spectra obtained from standard synthetic solutions of pure Hb(Fe(II)NO, MetHb, Hb(Fe(II))O2, and deoxy-Hb were used to determine the fractional content of MetHb and Hb(Fe(II))NO (6). In addition, Hb(Fe(II))NO and SNO-Hb were measured by the photolysis-chemiluminescence method (7).

**Bohr Effect in SNO-Hb—**SNO-Hb and Hb solutions at varying pH were prepared by dilution in PBS solutions pre-titrated to the specified pH with either NaOH or HCl. In the case of the acidification of PBS to achieve pH 6.80, the added concentration of chloride ion (an allosteric effector) was ~1% of the total anion concentration of PBS maintained at pH 7.40.

**Allosteric Effects of Organic and Inorganic Phosphates—**Hb or SNO-Hb prepared as described above was diluted in PBS solutions (pH 7.40) of monobasic and dibasic sodium phosphate to yield a final P042− concentration of either 10 or 100 mM. The change in anion concentration (specifically [Cl−]) with pH adjustment of these solutions was estimated to be negligible. Insolubile hexaphosphate (150 μM), 2,3-diphosphoglycerate (150 μM), or β-NADPH (Calbiochem) was dissolved in Hb buffer and adjusted to the specified pH before the addition of Hb/SNO-Hb.

**Transnitration and O2 Affinity: Influence of GSH on O2 Equilibria of SNO-Hb—**The O2 equilibria of SNO-Hb (1.8 SNO/tetramer) was compared in the presence and absence of varying concentrations of GSH to test the ability of a thiold acceptor to facilitate denitrosylation of SNO-Hb and, consequently, the offloading of GSH as a function of the O2 linked allosteric transition in Hb. In experiments in which GSH was present, the oxidation of hemes routinely observed with the oxygenation-deoxygenation process was accelerated. The formation of MetHb was minimized by using purified stroma-free Hb (PSF-Hb), a partially purified hemolysate containing physiological levels of catalase, superoxide dismutase, methemoglobin reductase, and HbA2. The hemolyzate was supplied by Biochemists and deoxy-Hb was maintained in a stopped reaction and added to Hb solutions containing 5 mM NADPH (naturally present in the red blood cell), including to provide reducing equivalents, but also functioning as an allosteric effector of the organic phosphate class (40, 41).

**Bioactivity Assay—**Thoracic aortic rings were harvested from New Zealand White rabbits as described previously (5). The rings were mounted on stirrups in tissue baths filled with Krebs-Henseleit buffer, pH 7.40 (no chelating agent), bubbled with either 21% O2, 5% CO2, and 78% N2, and then equilibrated with either 95% air or 95% N2 (i.e. pH 7.40). Rigorous deoxygenation was accomplished through several cycles of alternate flushing with ultra-high purity argon gas and applying a measured vacuum. The tonometer was mechanically rotated for 7 min in a 20 °C water bath, and a spectrum was obtained. A second round of deoxygenation was performed to verify the completeness of conversion to deoxy-Hb and its stability. An airtight syringe (Hamilton, Ontario, Canada) was then used to incrementally deliver measured volumes of room air into the tonometer through a rubber septum. Following each injection, the tonometer was rotated in a temperature-controlled (20 °C) water bath for 7 min to allow equilibration between the head space (~300 ml) and the Hb solution (3 ml), and then a visible spectrum was obtained. Injections of air were repeated until a fully oxygenated spectrum was observed.
balance N\textsubscript{2} (“normoxia”) or 5% CO\textsubscript{2} and balance N\textsubscript{2} (“hypoxia”; <1% O\textsubscript{2}), and maintained at 37 °C. This buffer contains ~125 mM Cl\textsuperscript{−} and ~1 mM PO\textsubscript{4}\textsuperscript{3−}. Changes in isometric tension were measured and recorded with Statham pressure transducers and a Grass polygraph. Phenylephrine was added to the tissue baths in concentrations sufficient to elicit an isometric tension response to acetylcholine. In some experiments, reduced GSH was added to the tissue baths 15 min prior to the administration of SNO-Hb. Responses to SNO-Hb are expressed as the percent change from the initial tension achieved with phenylephrine.

RESULTS

Oxygen Affinity of SNO-Hb: Influence of SNO Content—The influence of the extent of S-nitrosylation of Hb on O\textsubscript{2} affinity in SNO-Hb was studied in O\textsubscript{2}-binding experiments using solutions of Hb S-nitrosylated to varying degrees. As outlined above, the extent of Hb S-nitrosylation was varied by arresting the incubation of Hb with S-nitrosocysteine at predetermined points in time. The O\textsubscript{2}-binding curve for SNO-Hb was shifted leftward relative to that of Hb\textsubscript{A\textsubscript{0}} (Fig. 1A). When expressed in the form of the Hill plot (log pO\textsubscript{2} \textit{versus} log[Y/(1 − Y)]), where \(Y\) is the fractional O\textsubscript{2} saturation, it becomes clear that the leftward shift for SNO-Hb is asymmetric in that differences in affinity are most pronounced at low pO\textsubscript{2} values (Fig. 1B). Accordingly, the \(n_{50}\) (the slope of the Hill plot at half-maximal O\textsubscript{2} saturation, a measure of cooperativity) is lower in SNO-Hb than in Hb\textsubscript{A\textsubscript{0}} (Table I). Oxygen affinity rises (and \(P_{50}\) decreases) as a function of SNO content across the full range from 0 to (the maximal) 2 SNO groups/Hb tetramer (Fig. 1C), in contrast to the findings of Patel et al. (36) and in agreement with those of Bonaventura et al. (42). The relationship between O\textsubscript{2} affinity (defined as \(P_{50}\)) and SNO content appears to be parabolic, nearly linear shape (Fig. 1C).

Bohr Effect in SNO-Hb—The effect of varying pH on O\textsubscript{2}-binding equilibria was compared in Hb and SNO-Hb (SNO/Hb ratio = 1.72 ± 0.05) in PBS. Progressive increases in pH in the range from 6.80 to 8.00 shifted the O\textsubscript{2}-binding curves for both Hb\textsubscript{A\textsubscript{0}} and SNO-Hb leftward to similar degrees (Fig. 2). At each pH studied, the O\textsubscript{2} affinity of S-nitrosylated Hb was greater than that of unmodified Hb\textsubscript{A\textsubscript{0}}, although at pH 8.0, the difference between the two molecules was diminished (Fig. 2C). The magnitude of the pH dependence of O\textsubscript{2} affinity, expressed as the slope of log \(P_{50}\) \textit{versus} pH, was similar for the two molecules (Fig. 2C).

Comparative Allosteric Effects of Organic and Inorganic Phosphates on Hb and SNO-Hb—Phosphate anion concentrations have been recently shown to dramatically affect the propensity for NO addition \textit{versus} oxidation in reactions with hemoglobin, and an allosteric mechanism was proposed (6). Increasing the concentration of phosphate from 10 to 100 mM (in sodium phosphate buffers) indeed shifted the O\textsubscript{2}-binding curves to the right in Hb and to a similar extent in SNO-Hb (Fig. 3A), \textit{i.e.} both are similarly responsive to allosteric effectors. The \(n_{50}\) was increased slightly in SNO-Hb by organic and inorganic phosphate buffers, whereas it was decreased slightly in some instances in native Hb (Table I). In the presence of the organic phosphates 2,3-diphosphoglycerate and inositol hexaphosphate, the \(P_{50}\) values for Hb and SNO-Hb (in 0.1 M Hepes) were raised to similar extents as a function of pH (Fig. 3B).

Transnitrosation, Iron Nitrosyl Formation, and O\textsubscript{2} Affinity: Influence of GSH on O\textsubscript{2} Equilibria of SNO-Hb—The O\textsubscript{2}-binding curve for S-nitrosylated PSF-Hb was similar in character to that of S-nitrosylated Hb\textsubscript{A\textsubscript{0}} (Fig. 4A) and was shifted to the left relative to that of unmodified PSF-Hb (data not shown). O\textsubscript{2}-binding curves for S-nitrosylated PSF-Hb and PSF-Hb were both shifted rightward in the presence of NADPH (data not shown). The \(\Delta\log P_{50}\) between S-nitrosylated and unmodified PSF-Hbs was similar to that between S-nitrosylated Hb\textsubscript{A\textsubscript{0}} and unmodified Hb\textsubscript{A\textsubscript{0}}. When 1.5 mM GSH was also present during the deoxygenation-reoxygenation cycle, O\textsubscript{2} equilibria were further displaced to the right relative to those obtained in the absence of GSH (Fig. 4A). Yields of Hb(Fe(II))NO upon deoxy- genation in these experiments were ~30% of total [Hb] when

| TABLE I |
|---------|
| \(n_{50}\) (slope of the Hill plot at half-saturation, a measure of cooperativity) as a function of pH in Hb and SNO-Hb |
| \(\text{Hb}\) |
| \(\text{SNO-Hb}\) |
| pH 6.8 | pH 7.4 | pH 8.0 | pH 6.8 | pH 7.4 | pH 8.0 |
| Hb | 3.0 | 3.1 | 3.0 | 1.8 | 1.9 | 1.9 |
| Hb + DPG | 2.9 | 3.2 | 3.2 | 2.2 | 2.4 | 2.2 |
| Hb + IHP | 2.3 | 2.3 | 3.0 | 2.1 | 2.3 | 2.2 |

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measured by spectral deconvolution (6) and ~27% when measured by photolysis-chemiluminescence (Fig. 4B). Moreover, the amount of NO remaining bound to Hb in the deoxy state was only ~40% of that in the oxy state (Fig. 4B), showing that transnitrosation/denitrosylation reactions are favored in low $pO_2$.

15 mM GSH caused a leftward shift in $O_2$-binding compared with that obtained in the presence of 1.5 mM (Fig. 4A). This seemingly paradoxical normalization of the $O_2$-binding curve at very high GSH/SNO-Hb ratios was associated with and caused in significant part by GSH-induced heme oxidation of SNO-Hb during the deoxygenation-reoxygenation cycle (see “Discussion”).

Influence of GSH Concentration and $O_2$ Tension on the Vasoreactivity of SNO-Hb—Vascular responses to SNO-Hb were studied in aortic rings exposed to varying final concentrations of reduced GSH under both 21% $O_2$ and low $pO_2$ conditions that more closely simulate the tissues. The addition of GSH alone produced no change in tension in the majority of experiments; in ~10% of vessel rings, a small but transient vasorelaxant response was seen.

Under normoxic conditions, SNO-Hb (1.7 SNO groups/tetramer; $10^{-5}$ to $10^{-5.5}$ M Hb) elicited concentration-dependent, sustained increases in vessel tension as described previously (Fig. 5A) (1, 5). These vasoconstrictor responses were preserved in the presence of $10^{-7}$ to $10^{-5}$ M GSH (Fig. 5A). At $10^{-4}$ M GSH, vasoactive responses to SNO-Hb were variable, with relaxation observed in some preparations and blunted vasoconstriction in others (Fig. 5A). Vasorelaxation to SNO-Hb was, however, observed in the presence of millimolar (10$^{-3}$ M) GSH (Fig. 5B). Relaxation began 3–10 s after the addition of SNO-Hb to the tissue bath, consistent with response times to other agents in this system (data not shown).

In vessel rings made hypoxic (<1% $O_2$), SNO-Hb similarly produced dose-dependent contractions. However, the addition of glutathione even at low micromolar concentrations elicited relaxations (Fig. 5B). Specifically, vasoconstriction to SNO-Hb ($10^{-7}$ to $10^{-3}$ M) was observed in the presence of up to $10^{-6}$ M GSH, whereas in vessels exposed to $10^{-5}$ to $10^{-3}$ M GSH, SNO-Hb elicited potent vasorelaxation (Fig. 5B). At millimolar glutathione, SNO-Hb relaxation in hypoxia was comparable to that seen in normoxia (Fig. 5A), an indication that sensitivity of vessels to SNO-induced relaxation is not greater under hypoxic conditions, as we have previously reported (5). The dose-response curves for SNO-Hb in the presence of millimolar GSH were very similar (almost superimposable) to the curves generated by estimated amounts of GSNO formed in the reactions, whereas NO solutions were inactive at these concentrations (data not shown), i.e. GSNO, not NO as recently suggested (23), is responsible for relaxation in this system; NO is inactive.
The biological function of Hb Cys<sup>b93</sup>, which is highly conserved evolutionarily among higher animals, had remained obscure until recently. Several studies (1, 2, 5, 7) have now made the case that it “acts as an allosterically controlled NO buffer” (8), exchanging the NO group with ambient thiols, thereby producing vasorelaxant GSNO. For obvious reasons, bioassays can only reveal shifts in the position of this equilibrium by working under conditions (Fig. 4; see “Discussion”). This condition was not met in a recent study (23).

**DISCUSSION**

The biological function of Hb Cys<sup>b93</sup> is largely unknown. It is located in a protected pocket within the R conformation (5). Transnitrosation to glutathione is therefore disfavored in the R state relative to the T state. Allosteric modulators that shift the position of the R/T equilibrium toward R would therefore lower the yield of SNO-Hb transnitrosation reactions and decrease the bioactivity (Equation 4 and Fig. 5). At sufficiently high glutathione concentrations, however, Equation 2 indicates that GSNO formation will ultimately prevail. Indeed, our bioassay results confirm the formation of GSNO in the presence of glutathione, ascorbate, and other naturally occurring reductants (45). GSNO is highly reactive, liberating NO (46–50). This momentarily freed NO would be quickly captured at vacant hemes at a nearly diffusion-limited rate (2, 6). Hb thus limits the net release of NO and thereby regulates related bioactivity.

The interaction of SNO-Hb with GSH entails not only a transnitrosation reaction, but an additional process in which the NO group is reductively transferred to the heme presum-
ably via the transient release of NO (Fig. 4B). Under experimental conditions in which all hemoglobin molecules carry ~2 SNO groups and approach full oxygenation, an inevitable consequence is MetHb formation. With NO/Hb ratios in the physiological range, however, the NO addition reaction, which yields Hb(Fe(II))NO, outcompetes the MetHb-forming oxidation reaction (6) because the concentration of NO is significantly lower than the concentration of vacant (deoxygenated) hemes in HbO₂, and the binding of NO to HbO₂ is cooperative (6). Thus, a major product of GSH interaction with SNO-Hb in vivo is likely to be iron nitrosylhemoglobin (Equation 5).

\[
\text{SNO-Hb(Fe(II))/O}_2 + \text{GSH} + e^- \leftrightarrow \text{HS-Hb(Fe(II))/NO} + \text{GS}^- + O_2 \quad \text{(Eq. 5)}
\]

Taken together, these reactions present a scenario where the majority of NO/SNO that wanders from Hb is recaptured and thereby prevented from leaving the red blood cell. SNO-Hb exists in equilibrium with Hb(Fe(II))/NO (Equations 1 and 5).² The position of this redox-coupled equilibrium is linked to the allosteric state of Hb as evidenced here by SNO-to-heme iron transfer of NO upon deoxygenation. It has been previously inferred from O₂-binding studies that this amount of Hb(Fe(II))/NO is very small and inconsequential (36). In these earlier studies, however, SNO-Hb was subjected to deoxygennation in the absence of a reductant. Under such artificial conditions, SNO-Hb would be restricted to an unfavorable homolytic decomposition reaction (that yields NO’ and Hb thyl radical, ‘S-Hb(Fe(II))). Moreover, even if a reductant were present, the supraphysiological ratios of NO to heme in this system could retard the transition to T structure upon deoxygenation as NO replaces the departed O₂ ligands. In this study, we have shown that the addition of physiological reducing agents (NADPH, GSH) facilitates NO migration to thiols from heme and does so to a greater extent at low pO₂ (i.e. in T structure). Specifically, a major fraction of SNO-Hb(Fe(II))/O₂ is converted into Hb(Fe(II))/NO upon deoxygenation. Thus, both the activity-generating transnitrosation reaction that yields vasorelaxant S-nitrosothiol (Equation 4) and the reductive NO-storing reaction yielding Hb(Fe(II))/NO (Equations 1 and 5) are facilitated by deoxygenation.

SNO-Hb oxygen-binding curves exhibit diametrically opposite effects at different concentrations of GSH (15 mm versus 1.5 mm) (Fig. 4). This behavior may be related to differing amounts of mixed disulfide, MetHb, and iron nitrosylhemoglobin, the formation of which is glutathione-dependent. To control these effects, we used a purified stroma-free red blood cell hemolytic processing work by Chan consistent with the modeling work of Stamler et al. Furthermore, the existence of S-nitrosohemoglobin (Equation 4) is the reason for this discrepancy is unclear. Wolzt et al. (23) have reported that additional thiols can be modified in Hb and that molecules with >2 SNO groups might have different functional behavior; however, the existence of >2 SNO groups/tetramer probably reflects disruption of Hb tertiary/tetrameric structure by high concentrations of thiol reactants (23, 54). There may indeed be a diversity of SNO-Hb forms and functions, but this diversity will be primarily linked to homotropic interactions.

SNO-Hb and iron nitrosylhemoglobin from blood reveal 1 of each per 1000 tetramers (1, 5). The increases in O₂ affinity produced by S-nitrosylation are thus unlikely to affect the O₂-binding properties of blood. More generally, the same holds true for iron nitrosylhemoglobin, which has been reported to decrease O₂ affinity (39), but again, only at supraphysiological NO concentrations and then only under non-physiological conditions (low pH, added inositol hexaphosphate, low temperature). The importance of NO group binding to hemes and thiols of Hb lies not in an effect on the population of Hb at large, but on the functional behavior of molecules carrying the NO, i.e. S-nitrosoylation may serve to conserve SNO by favoring the R structure (as discussed above), whereas migration to hemes limits the escape of NO upon deoxygenation of SNO-carrying molecules. In this way, Hb tightly regulates the dispensing of NO groups. Since blood flow is regulated by nanomolar SNO targeted to smooth muscle (1, 5, 32–34, 44), this dispensing of NO groups (for the purpose of dilating blood vessels) is far more important for O₂ delivery than any NO-related change in O₂ saturation of Hb, which could only be effected by supraphysiological NO levels.

The O₂-binding properties of SNO-Hb, like those of unmodified Hb, are strongly pH-dependent. The magnitude of the Bohr effect in SNO-Hb is similar to that seen with unmodified Hb. Specifically, the sensitivity of SNO-Hb to changes in pH is similar to that of native Hb. The ability of His¹⁴⁶ to bind protons is reported to account for nearly one-half of the alkaline Bohr effect in Hb (55–57). Our findings therefore suggest that the behavior of the salt bridge between His¹⁴⁶ and Asp³⁴ is not altered by S-nitrosoylation of the protein. This suggestion is consistent with the modeling work of Stamler et al. (5). Modelling work by Chan et al. (18), on the other hand, implied that these terminal residues would be disordered in the T as well as R states of SNO-Hb, thus ruling out a significant contribution to the Bohr effect by these residues. We have reasoned that the formation of this salt bridge will increase the pK of Cys¹⁰³ in Hb (5), i.e. protonation of His¹⁴⁶ and Cys³⁴ is linked. It remains to be seen, therefore, if Cys¹⁰³ contributes to the Bohr effect, in which case, protonation of nitrosocysteine β93 may facilitate NO group release (58). The O₂ affinity of SNO-Hb is also regulated by phosphate anion concentration (Fig. 3), in keeping with the recent observation that NO chemistry with Hb can be greatly influenced by the buffers commonly used for studies of Hb function (6).

The thermodynamic implications of Monod’s concept of allostery in the regulation of protein function were developed by Wyman (13, 14), with hemoglobin as the model. Numerous reciprocal relationships between O₂ equilibria and the binding of heterotropic allosteric effectors, required by thermodynamics, have been demonstrated experimentally. For example, the covalent binding of CO₂ to the amino terminus of Hb α-globin chains lowers O₂ affinity, and accordingly, O₂ binding at the hemes triggers the dissociation of CO₂ (the Haldane effect) (58). These linkage principles similarly illuminate the interaction of Hb with the NO group. Five groups have shown S-nitrosoylation

² B. P. Luchsinger, A. J. Gow, T. J. McMahon, J. S. Stamler, and D. J. Singel, manuscript in preparation.
to be favored in the R structure (11, 19, 22, 36, 42). Subsequently, we and two other groups (36, 42) have demonstrated the reciprocal effect of S-nitrosylation increasing the O₂ affinity, and we have also reported that heme deoxygenation promotes NO group release from SNO-Hb (1).

This linkage has implications for the recent report of Patel et al. (36) who determined that transnitrosation of GSH by SNO-Hb is more favorable in the R than T structure. Specifically, they indicated that the equilibrium constant for GSNO formation from SNO-Hb (Equations 2 and 4) is greater in the R structure and concluded that the O₂-dependence of the vasorelaxation could not involve a mechanism that is “intrinsic to thermodynamic linkage of O₂ ligation and of both groups are difficult to reconcile with the apparent SNO-Hb is linked to the allosteric transition. The conclusions of both groups are difficult to reconcile with the apparent thermodynamic linkage of O₂ ligation and S-nitrosylation.

These studies have a number of shortcomings. Patel et al. (36) did not actually evaluate either the aerobic or anaerobic equilibrium constants for Equation 2, nor did they actually measure the bioassay responses on which their biological conclusion rests. In fact, their conclusions rest upon a series of suppositions, estimates, and extrapolations that are unjustified. As a most notable and crucial example, they substantially overestimated, with no supporting argument or cited reference, the amounts of NO/SNO that would be needed to dilate blood vessels and substantially underestimated the circulation time of red blood cells.

Wolzt et al. (23) did conduct bioassays, but they worked under conditions in which Hb is significantly dissociated into dimers (see “Experimental Procedures”), which do not transition to T structure (38, 59). Their methods therefore prevented them from rendering any meaningful conclusion as to the allosteric-related effects of oxygen tension. Moreover, since Wolzt et al. used only high concentrations of glutathione, they would have masked (as we show in Fig. 4) the potentiating effect of the Hb quaternary structure (Equation 2). For obvious technical reasons, the organ chamber bioassay can only illuminate the reciprocal effect of the GSH-dependent relaxations of SNO-Hb are mediated by glutathione, but challenged the notion that the bioactivity of SNO-Hb is linked to the allosteric transition. The conclusions of both groups are difficult to reconcile with the apparent thermodynamic linkage of O₂ ligation and S-nitrosylation.

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Coupling of Oxygen Binding and Vasoactivity in SNO-Hb

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Functional Coupling of Oxygen Binding and Vasoactivity in S-Nitrosohemoglobin
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