Reaction of S-Nitrosoglutathione with the 
Heme Group of Deoxyhemoglobin*

Netanya Y. Spencer, Hong Zeng, Rakesh P. Patel‡, and Neil Hogg§

From the Biophysics Research Institute and Free Radical Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the Department of Pathology and Center for Free Radical Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

The mechanism of interaction between S-nitrosoglutathione (GSNO) and hemoglobin is a crucial component of hypotheses concerning the role played by S-nitrosohemoglobin in vivo. We previously demonstrated (Patel, R. P., Hogg, N., Spencer, N. Y., Kalyanaraman, B., Matalon, S., and Darley-Usmar, V. M. (1999) J. Biol. Chem. 274, 15487-15492) that transnitrosation between oxygenated hemoglobin and GSNO is a slow, reversible process, and that the reaction between GSNO and deoxygenated hemoglobin (deoxyHb) did not conform to second order reversible kinetics. In this study we have reinvestigated this reaction and show that GSNO reacts with deoxyHb to form glutathione, nitric oxide, and ferric hemoglobin. Nitric oxide formed from this reaction is immediately autocaptured to form nitrosylated hemoglobin. GSNO reduction by deoxyHb is essentially irreversible. The kinetics of this reaction depended upon the conformation of the protein, with more rapid kinetics occurring in the high oxygen affinity state (i.e. modification of the Cys9-93) than in the low oxygen affinity state (i.e. treatment with inositol hexaphosphate). A more rapid reaction occurred when deoxymyoglobin was used, further supporting the observation that the kinetics of reduction are directly proportional to oxygen affinity. This observation provides a mechanism for how deoxygenation of hemoglobin/myoglobin could facilitate nitric oxide release from S-nitrosothiols and represents a potential physiological mechanism of S-nitrosothiol metabolism.

The interaction between S-nitrosoglutathione (GSNO) and hemoglobin has become an area of intense interest due to the discovery that circulating erythrocytes contain measurable levels of S-nitrosohemoglobin (HbSNO) (1), and that the combination of S-nitrosohemoglobin and glutathione (GSH) will relax vessels in an oxygen sensitive manner (2). HbSNO is a post-translationally modified form of hemoglobin that contains a nitrosated cysteinyl residue at the β-93 position. The mechanism for the formation of HbSNO in vivo remains unknown, and the functional consequences of this modification are the subject of intense debate (1-4). It has been suggested that S-nitrosation of Hb represents an oxygen-sensitive mechanism to control vascular tone (1). The crux of this hypothesis is that transnitrosation, i.e. the chemical transfer of the nitroso group, between Hb and GSH is an oxygen-sensitive reaction in which deoxyoxygenation of Hb allows the eventual formation of GSNO, which may then act as a vasodilator. The mechanism by which intra-erythrocyte GSNO acts as a vasodilator is as yet unknown. In a new twist to this hypothesis it has recently been suggested that rapid transnitrosation to form GSNO would be fatal, due to a massive vasodilatory response upon deoxygenation of Hb (5). In order to avoid such an outcome the majority of the NO has been proposed to be autocaptured at the deoxygenated heme, to give nitrosyl hemoglobin (HbNO), in which nitric oxide is bound to the iron of ferrous hemoglobin. It has been additionally suggested that the transfer of “NO” from thiol to heme is reversible (5). In order to critically examine this hypothesis, we have undertaken a kinetic and mechanistic examination of the interaction of S-nitrosothiol with Hb. We previously demonstrated that S-nitrosation of Hb increases its oxygen affinity (3). Consequently the S-nitrosated Hb will only be deoxygenated after the majority of the unmodified Hb has released its oxygen. This suggests that oxygen-dependent transnitrosation will only occur in situations where the erythrocyte is substantially deoxygenated. In addition, we examined the kinetics of the reaction between GSNO and Hb. The reaction between GSNO and oxygenated hemoglobin (oxyHb) conforms well to second order irreversible kinetics. The reaction is slow (~0.1 M s⁻¹) in both directions and has an equilibrium constant of 1.3 (3). We also demonstrated that the reaction between GSNO and deoxyoxidized hemoglobin (deoxyHb) is also slow but does not conform to second order reversible kinetics. Consequently a value for the equilibrium constant for transnitrosation between GSNO and deoxyHb cannot be calculated, and should not be inferred from our data as was done recently by McMahon et al. (5).

In this study we have re-examined the reaction between GSNO and deoxyHb and conclude that GSNO can be directly reduced by deoxyHb to form GSH, metHb, and nitric oxide. The nitric oxide may then bind to the free heme of hemoglobin to form HbNO. This reaction is essentially irreversible.

EXPERIMENTAL PROCEDURES

Materials—GSH, sodium nitrite, N-ethylmaleimide (NEM), bovine serum albumin, phytic acid (inositol hexaphosphate, IP₆), and myoglobin (Mb) from horse heart were obtained from Sigma. Diethylenetriaminepentacetic acid (DTPA) was obtained from Fluka. Human hemoglobin was prepared from freshly drawn blood as previously described (6). GSNO was synthesized from the reaction between GSH and nitric oxide by the addition of

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‡ To whom correspondence should be addressed: Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Rd., P.O. Box 26509, Milwaukee, WI 53226. Tel: 414-456-4012; Fax: 414-456-5142; E-mail: nhogg@post.itas.mcw.edu.

§ To whom correspondence should be addressed: Biophysics Research Institute, Medical College of Wisconsin, 8701 Watertown Plank Rd., P.O. Box 26509, Milwaukee, WI 53226.

1 The abbreviations used are: GSNO, S-nitrosoglutathione; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; metHb, ferric hemoglobin; HbNO, nitrosyl hemoglobin; HbSNO, S-nitrosohemoglobin; NO, nitric oxide; GSH, glutathione; NEM, N-ethylmaleimide; DTPA, diethylenetriaminepentacetic acid; Mb, myoglobin; MbNO, nitroso myoglobin; IP₆, inositol hexaphosphate; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; EPR, electron paramagnetic resonance.

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and acidified sodium nitrite as previously described (7, 8). ApoHb and methHb were prepared from hemoglobin as previously described (6).

Hemoglobin was deoxygenated, and the various solutions used in the experiments were rendered anaerobic, by alternating vacuum and argon gas. The solution was first placed in a gas-tight glass vial which was sealed with a rubber stopper. Then argon gas and vacuum were alternately applied to the inside of the vial through a needle for at least 10 min, with five or more cycles of argon and vacuum. No room air was allowed to enter the vial at any time during this treatment. Once fully deoxygenated, samples were transferred to an anaerobic glove box (Coy Laboratory Products, Inc.) to allow experiments to be carried out under anaerobic conditions. All experiments were performed at 24 °C in subdued light. Samples were removed at various time points and filtered using Centricon filtration devices under anaerobic conditions. The filtrate was then assayed for GSH (●), GSNO (▲), and total glutathione (○). Data points represent mean ± S.E. (n = 3).

In some experiments, hemoglobin thiols were blocked by preincubation with excess NEM. In order to measure GSH, it was necessary to remove excess NEM by passing down a Sephadex G-25 column. Fractions were collected, and only those fractions not containing NEM (as determined by UV-visible spectrophotometry) were combined and concentrated using filter devices. Thiol measurements indicated that NEM treatment decreased hemoglobin thiol content from 84% to 0%.

HPLC Detection of S-Nitrosothiols and Glutathione—S-Nitrosothiols and glutathione (9, 10) were analyzed and quantified by HPLC as previously described.

Chemiluminescence Detection of Nitrite, Nitrate, and S-Nitrosothiols—Following the manufacturer's standard protocols, a Sievers 280 Nitric Oxide Analyzer was used to detect nitrite and nitrate. To detect S-nitrosothiols, cupric sulfate (1 mM) was included in the standard nitrite-detecting solution (7.1 mg/ml KI, 76% glacial acetic acid) in the purge vessel.

Electron Paramagnetic Resonance Studies—EPR spectra were obtained using an X-band spectrometer with the following conditions: modulation amplitude: 2.5 G, time constant: 0.128 s, scan time: 1 min, frequency: 9.17 GHz, microwave power: 10 mW, center field: 3260 G, scan width: 500 G, sample temperature: 77 K, signal averaging: 4. EPR spectra of nitrosylHb were quantified by double integration and comparison with standard spectra of nitrosylHb collected under identical conditions.

RESULTS

DeoxyHb (833 μM, all concentrations are expressed in terms of heme) was incubated with GSNO (approximately 20 μM) in phosphate buffer containing DTPA, in an anaerobic glove box, in subdued light. Samples were removed at various time points during an hour time course, and then subjected to ultrafiltration under anaerobic conditions. A slow decrease in the concentration of GSNO in the eluates was observed by chemiluminescence (Fig. 1). The percent decrease in [GSNO] was variable between experiments, as shown by the large error bars in Fig. 1. GSNO disappearance was associated with the formation of a small amount of GSH. Treatment of the eluates with dithiothreitol caused reduction of all GSNO and any GSSG which may have been present, and accounted for the discrepancy between the large amount of GSNO lost and the somewhat smaller amount of GSH formed. Controls using either phosphate buffer (containing DTPA) or double-distilled water, instead of hemoglobin, did not promote any loss of GSNO over the entire time course (data not shown).

Because deoxyHb-dependent loss of GSNO might be attributed to a transnitrosation reaction to the hemoglobin Cys-β93, a similar experiment to that shown in Fig. 1 was performed using NEM-pretreated hemoglobin. NEM pretreatment completely modified all 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)-detectable thiol groups. When NEM-pretreated deoxyHb (833 μM) was incubated with GSNO (21 μM), again the decomposition of GSNO was observed (Fig. 2). As with untreated hemoglobin, a concomitant formation of GSH was observed. This indicates that, at least in this case, transnitrosation cannot be responsible for the formation of GSH from GSNO. When fully oxygenated hemoglobin with excess NEM was used, there was no loss of GSNO over the same length of time (data not shown).

In addition to modifying the thiol residues of hemoglobin, NEM modification of the β93 cysteiny1 residue also stabilizes hemoglobin in the so-called “R”-state or high oxygen-affinity state (11). The rates of GSNO decay and GSH formation were slightly faster when NEM-treated hemoglobin was used, suggesting that the kinetics of this reaction are related to the conformation of the hemoglobin tetramer. In order to test this hypothesis we examined the kinetics of GSNO decay and GSH formation in the presence of IP6, which stabilizes hemoglobin in the low oxygen-affinity “T” conformation. As shown in Fig. 3, the presence of IP6 resulted in both a slower loss of GSNO and a slower formation of GSH when compared with NEM Hb (Fig. 2). These data indicate that the rate of reaction of GSNO with hemoglobin depends on the conformation of the hemoglobin and therefore on the degree of oxygen affinity.

Nitrile/nitrate analysis by chemiluminescence indicated that the nitroso group that had been displaced from GSNO did not appear in an ultrafiltrate as an oxide of nitrogen (data not shown). However, electron paramagnetic resonance (EPR) analysis revealed the concomitant formation of HbNO. As shown in Fig. 4A, incubation of 0.5 mM GSNO with 0.5 mM hemoglobin, with excess NEM present, resulted in the formation of an EPR spectrum indicative of HbNO showing a small contribution from 5-coordinate heme and a large contribution...
from 6-coordinate heme (12). The magnitude of the EPR signal increased as a function of time. In the presence of IP_6, but not NEM, only the 5-coordinate signal was observed and the signal evolution was slowed (Fig. 4B). The 5-coordinate heme signal has a clear nitrogen hyperfine (a^N = 16.75 G). This signal has previously been attributed to nitric oxide bound to the ferrous iron of the α-subunit heme, causing a displacement of the proximal histidine ligand (12, 13). These data indicate that the reaction between GSNO and hemoglobin yields both GSH and nitric oxide, the latter of which rapidly binds to unliganded ferrous heme.

To convert GSNO to GSH and nitric oxide it is necessary to reduce the S-nitrosothiol by one electron. It seemed likely that this electron could come from the ferrous heme iron, thereby oxidizing it to ferric iron. Consequently, UV-vis spectrophotometry was used to detect the formation of the ferric form of hemoglobin (metHb) during the reaction between GSNO and deoxyHb under anaerobic conditions, in the presence of either NEM or IP_6. The reaction was effectively stopped at various time points by 1:10 dilution with aerobic 50 mM phosphate buffer containing DTPA, and the samples were immediately scanned in the spectrophotometer. The absorbance at 630 nm was observed to increase over time faster with NEM present than with IP_6. Quantitative comparison of the rate of metHb formation to that of HbNO formation (from Fig. 4) indicated that the rates of formation of metHb and HbNO follow very similar time courses, suggesting that their formation occurs as a result of the same kinetic process in an almost 1:1 stoichiometry (Fig. 5). It is unlikely that the metHb measured in these experiments is formed from the reaction between oxygen and HbNO, because the reaction between oxygen and HbNO to form metHb is very slow. No metHb was formed over the same time in the absence of GSNO (data not shown) indicating that no significant autoxidation occurs under the experimental conditions employed in these studies.

To further investigate interactions of hemoglobin and GSNO, another experiment was performed in which fully oxygenated hemoglobin (500 μM) was incubated with GSNO (500 μM) for 18 h in the dark in the presence of DTPA (Fig. 6A, inset). At various time points during this incubation, aliquots were removed and separated by ultrafiltration. A slow disappearance of GSNO from the eluates was observed over this time. This was attributed to either transnitrosation or S-thiolation (1, 4). No HbNO was observed during this time (Fig. 6A, inset). After 18 h, the solution was thoroughly deoxygenated. Over the next 3 h, several more aliquots were removed and separated. Both the disappearance of GSNO and the formation of HbNO were followed. As shown in Fig. 6A, the loss of GSNO was paralleled by the formation of HbNO, suggesting that all the HbNO observed occurs as a result of the decay of GSNO in solution.

After 3 h, the solution was re-exposed to oxygen (Fig. 6A, arrow), and over the next 9.5 h, several aliquots were removed and either separated by ultrafiltration or examined by EPR. During this time, the GSNO level in the eluates continued to decrease. The HbNO signal did not change during the first hour in the presence of oxygen, but decreased slowly over the total 9.5 h. These observations indicate that the formation of nitrosyl-Hb from GSNO is irreversible and suggest that transnitrosation from deoxyHbHSNO to GSH is much slower than the reaction between GSNO and deoxyHb.

The EPR spectrum of HbNO taken from the experiment shown in Fig. 6A after incubation for 180 min in an anaerobic environment consisted of mainly 5-coordinate heme with a small contribution from 6-coordinate heme (Fig. 6B). The spectra were representative of three independent experiments. B, EPR spectra of HbNO with excess IP_6, GSNO (500 μM) and deoxyHb (500 μM) were incubated for 120 min. Aliquots were removed at 5, 20, 60, and 120 min and immediately frozen in liquid nitrogen for spectral analysis. Spectra are representative of three independent experiments.
large contribution of 6-coordinate heme in Fig. 6B, spectrum A, is likely due to the fact that the hemoglobin thiol has been extensively modified by either S-nitrosation or S-thiolation (cf. NEM-treated hemoglobin in Fig. 4A). This observation indicates that the Cys-93 thiol has been modified during the 18 h in room air, either by transnitrosation or S-thiolation, and that deoxygenation did not appreciably reverse the thiol modification, even over 3 h. Upon reintroduction of oxygen to the sample shown in Fig. 6B, spectrum A, the hyperfine structure was lost but there was no change in nitrosyl Hb concentration calculated from the double integrated area of the spectra.

In order to examine if GSNO could also react with myoglobin, a heme protein with a very high oxygen affinity, GSNO was incubated with myoglobin, and nitrosoglutathione (M(NOS)) formation was examined by EPR. The formation of both metMb (not shown) and MbNO was observed. The formation of MbNO (Fig. 7) occurred more rapidly than did the formation of NEM-treated HbNO (Fig. 4A), with 114 μM MbNO formed after only 45 min. As myoglobin does not contain an accessible cysteine residue, transnitrosation could not be responsible for this observation. This again suggests that the kinetics of reaction between GSNO and heme groups are related to oxygen accessibility.

Previously we demonstrated that, using trichloroacetic acid precipitation, an accurate determination of the kinetics of transnitrosation between oxyHb and GSNO could be obtained (3). However, such a determination was impossible with deoxyHb due to the fact that some of the GSNO was consumed immediately after initiation of the reaction. As shown in Fig. 8, a portion of the GSNO is lost immediately upon the treatment of deoxyHb, but not oxyHb, with GSNO, followed by trichloroacetic acid precipitation. The amount lost is proportional to the Hb concentration and is not affected by the presence of NEM. It originally appeared as though GSNO was binding to the heme of Hb and was co-precipitating with the protein as this effect was not observed with apoHb, metHb, or bovine serum albumin (data not shown). We now know that the loss of GSNO was due to the direct reaction between heme and GSNO detailed in this study. These results indicate that when trichloroacetic acid precipitation denatures hemoglobin, the heme pocket is completely opened, allowing rapid reduction of GSNO. This is a potential problem for the measurement of GSNO in the presence of hemoglobin under anaerobic conditions using trichloroacetic acid precipitation.

**DISCUSSION**

In this study, we have observed a previously unknown reaction between S-nitrosoglutathione and deoxyhemoglobin. When GSNO is incubated with deoxyhemoglobin, GSH is formed from the GSNO. There are at least two possible reactions that could describe the mechanism of formation of GSH from GSNO. The first, transnitrosation, involves transfer of the nitroso group from GSNO to the Cys-β3 residues of hemoglobin, as shown in Equation 1. The second possibility is reduction of GSNO by one electron in a reaction that does not involve the thiol of Cys-β3, as shown in Equation 2.

\[
\text{GSNO} + \text{HbSH} \rightarrow \text{HbSNO} + \text{GSH} \quad \text{(Eq. 1)}
\]

\[
\text{GSNO} + e^- \rightarrow \text{GSH} + \text{NO} \quad \text{(Eq. 2)}
\]

To distinguish between these two possibilities, NEM was used to block all the Cys-β3 thiols in order to prevent any transnitrosation reactions. If transnitrosation appreciably contributes to the formation of GSH from GSNO, blocking the
thiols would slow or stop GSH formation. Surprisingly, the rate of formation of GSH was not inhibited. Therefore, the possibility of transnitrosation was excluded and a reductive mechanism was favored (Equation 2).

It has been shown that when NEM binds the Cys-@93 thiol, the conformation of the hemoglobin molecule is changed. Specifically, the hemoglobin is forced into the R (high oxygen affinity) state when these thiols are modified. It seemed possible that the conformation of the globin protein could affect the rate of formation of GSH from GSNO. To test this possibility, we treated hemoglobin with IP6, which stabilizes the low-oxygen-affinity T state when these thiols are modified. It seemed possible that the conformation of the hemoglobin molecule is changed. Specifically, the heme is deoxygenated, yet the globin protein is in the high oxygen affinity R-state.

Since GSNO was converted to GSH without a transnitrosation reaction to form HbSNO, it became necessary to determine the fate of the nitroso group of GSNO. If the nitroso group were converted to nitrite or nitrate, it should have been present in the ultrafiltrates. However, neither nitrite nor nitrate was detectable by chemiluminescence. Therefore, it appeared that the nitroso group had become associated with the protein, but not with the Cys-@93 thiol. This was confirmed by EPR as the formation of HbNO was observed. In agreement with GSH formation, the rate of HbNO formation was enhanced by the presence of NEM and was diminished by the presence of IP6. The observation that HbNO was formed along with GSH indicated that a one-electron reduction of GSNO had occurred (Equation 2) and that released nitric oxide had been rapidly captured by deoxyHb. One possible source of an electron was the ferrous heme iron. If ferrous heme iron were to donate the electron, the result would be stoichiometric metHb formation.

To test this possibility, we examined metHb formation by UV-visible spectroscopy. As predicted, metHb was formed when GSNO and deoxyHb were incubated together. Bonaventura et al. (14) also observed significant metHb formation during the reoxygenation of HbSNO in the presence of glutathione, which they ascribed to nitric oxide formation but which could have arisen from the direct oxidation of heme iron by GSNO. IP6 slowed the rate of formation of metHb, whereas NEM increased it (Fig. 5). The stoichiometry between metHb formation and HbSNO formation was almost 1:1. The overall reaction can be thought of as taking place in two steps. First, heme iron is oxidized by GSNO to form GSH, metHb, and nitric oxide (Equation 3). Second, the nascent nitric oxide reacts with another unliganded heme to form HbNO (Equation 4). The net reaction is shown in Equation 5.

\[
\text{Hb}^{\text{Fe}^{3+}} + \text{GSNO} + \text{H}^+ \rightarrow \text{Hb}^{\text{Fe}^{3+}} + \text{GSH} + \text{NO} \quad (\text{Eq. 3})
\]

\[
\text{Hb}^{\text{Fe}^{2+}} + \text{NO} \rightarrow \text{Hb}^{\text{Fe}^{2+}} \text{NO} \quad (\text{Eq. 4})
\]

\[
2\text{Hb}^{\text{Fe}^{3+}} + \text{GSNO} + \text{H}^+ \rightarrow \text{Hb}^{\text{Fe}^{3+}} + \text{Hb}^{\text{Fe}^{3+}} \text{NO} + \text{GSH} \quad (\text{Eq. 5})
\]

Using the present methodology, we cannot distinguish whether the oxidation and nitrosylation reactions occur within one hemoglobin tetramer or whether the oxidation of one heme allows the formation of nitric oxide which then diffuses through solution to another hemoglobin tetramer to nitrosylate a heme therein.

Although this reaction does not involve the Cys-@93 thiols of hemoglobin, the rate of the reaction is affected by the modification of this thiol. When all free Cys-@93s are blocked with NEM, the reaction rate approximately doubles as compared with IP6-treated hemoglobin. We speculate that this is due to the fact that modification of this thiol forces the globin tetramer into the R (high oxygen affinity) state. When IP6 is used to force hemoglobin into the T-state, the reaction rate is diminished.

To test whether Equation 5 is reversible, the experiment shown in Fig. 6 was carried out. When GSNO was incubated with oxyHb, GSNO slowly decayed but no HbNO was formed. This confirms the fact that Equation 5 cannot occur with fully oxygenated hemoglobin. Because NEM was not present to block thiols, the decay of GSNO was attributed to transnitrosation (1, 3), S-thiolation (4), or both. Therefore, at the moment that this solution was deoxygenated after 18 h of aerobic incubation, a substantial number of Cys-@93 thiols will have been modified. Thus, these molecules would be stabilized in the R conformation, as we have previously shown (3). After deoxygenation, HbNO was formed from the reduction of the GSNO remaining in solution. The EPR spectrum of the HbNO indicated a large contribution from 6-coordinate heme, suggesting that a major portion of the HbNO molecules still contained a thiol modification after 3 h in an anaerobic state. Deoxygenation therefore did not appreciably reverse the thiol modifications. Upon re-introduction of oxygen, the concentration of HbNO remained constant for an hour indicating that the formation of HbNO was not reversible.

Our current hypothesis for the interaction of GSNO with hemoglobin is shown in Scheme 1. Oxygen binding to deoxyHb is a complex cooperative process. For the sake of simplicity we have shown this reaction as a simple reversible binding defined by rate constants \( k_1 \) and \( k_{-1} \). Oxygen binding to HbSNO is similarly treated and defined by rate constants \( k_3 \) and \( k_{-3} \). Transnitrosation reactions between GSNO and oxyHb or deoxyHb are defined by rate constants \( k_2 \) and \( k_{-2} \) or \( k_4 \) and \( k_{-4} \), respectively. We have previously measured \( k_2 = 0.13 \text{ M}^{-1} \text{s}^{-1} \) and \( k_{-2} = 0.1 \text{ M}^{-1} \text{s}^{-1} \), giving a value for the equilibrium constant \( K_2 = 1.3 (3) \). Rossi et al. (15) calculated a value of 0.36 \text{ M}^{-1} \text{s}^{-1} for \( k_2 \) and reported a value of 0.101 for \( k_{-2} \); however, this latter value is likely a measure of \( k_{-2} \) as the method they used followed GSNO formation. We also previously demonstrated that HbSNO has a higher oxygen affinity than Hb, which means that \( K_2 K_3 < 1 \) (3). Due to the principle of microscopic reversibility, \( k_1 K_2 K_3 = 1 \) and consequently \( k_{-1} K_{-2} K_{-3} > 1 \). This means that GSNO formation via transnitrosation will be thermodynamically favored under deoxygenated conditions, as has been previously indicated (2, 5). However, this analysis does not predict or constrain the kinetic parameters for the

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individual reaction steps, because thermodynamic favorability is not a predictor of reaction rate.

In our previous publication (3) we discussed the difficulties of measuring the rate of transnitrosation in the deoxy state. What at first appeared as binding of GSNO to deoxyHb we now appreciate is a rapid reaction between free heme and GSNO during trichloroacetic acid-dependent precipitation/denaturation. It should be stressed that this reaction is only an issue with unliganded heme and does not occur with oxyHb, validating our kinetic analysis under oxygenated conditions. The fact that this same reaction occurs with intact deoxyHb (reactions $k_5$ and $k_6$ in Scheme 1), albeit at a slower rate, and that GSNO slowly reacts with GSH ($k_7 = 8.3 \times 10^{-8} \text{M}^{-1} \text{s}^{-1}$) (9) indicates that the equilibrium “square” shown in Fig. 1 will never achieve equilibrium due to an irreversible loss of GSNO and ferrous Hb. It is possible, from Fig. 5, to approximate the value of $k_6$, assuming this reaction conforms to second order kinetics. This calculation gives a value of 0.03 M$^{-1} \text{s}^{-1}$ for the NEM-treated case and 0.01 M$^{-1} \text{s}^{-1}$ for the IP$_6$-treated case. It must be stressed, however, that a full kinetic analysis of these reactions has not yet been performed and these numbers must be taken as approximations. It can be postulated that the value of $k_6$ (i.e. the reaction between GSNO and the heme of HbSNO) will be similar to the rate constant for the NEM-treated case, as thiol modifications by both NEM and S-nitrosothiol have similar effects on oxygen affinity. Our data indicate that the reaction described by $k_5$ outcompetes that described by $k_4$, as the formation of metHb closely follows the formation of HbNO (Fig. 5), even in the absence of NEM.

These data can be extrapolated to a circulating erythrocyte, but only with caution. The S-nitroso group of oxyHbSNO will be in dynamic exchange with intracellular GSH. Assuming a starting point where all of the nitroso groups are associated either with GSH as GSNO, or else with oxyHb as oxyHbSNO, it would take several hours for this dynamic exchange to reach equilibrium. At equilibrium the concentration of oxyHbSNO and GSNO will be approximately equal ($K_1 = 1.3$ assuming the concentration of heme thiois and glutathione are the same). The reaction of GSNO with GSH ($k_7$) indicates that this equilibrium cannot be sustained indefinitely as the S-nitroso group will eventually be converted to nitroxy. The circulating erythrocyte is, however, rapidly and repeatedly passing through areas of low oxygen tension and so it is highly unlikely that equilibrium will ever be established. In areas of low oxygen tension, GSNO will react with the unliganded heme group to liberate nitric oxide and oxidize the hemoglobin to metHb, which can be re-reduced by the metHb reductase system of the erythrocyte. The nitric oxide can escape from the red cell, bind to deoxyHb, or react with oxyHb. It has recently been demonstrated that when partially oxygenated, binding of nitric oxide to deoxyHb is favored over reaction with oxyHb (16). Nitric oxide escape appears unlikely as the kinetic barriers that prevent nitric oxide diffusion into red cells will also have to be overcome on the way out (17). Consequently, the formation of HbNO is the most likely end point.

The reaction between GSNO and deoxyMb is faster than the reaction between GSNO and deoxyHb, which also agrees with our hypothesis that the rate of the reaction between heme and GSNO is proportional to oxygen affinity. This reaction may be of great significance as a route of intracellular GSNO metabolism in myoglobin-containing muscle cells. It has previously been demonstrated that nitrosylmyoglobin is formed during perfusion of the intact rat heart with GSNO (18). GSNO is a potent protective agent against reperfusion injury in this model and it is tempting to speculate that myocyte myoglobin may play a role in the release of nitric oxide from GSNO (19, 20).

In conclusion, the direct reduction of GSNO by the heme group of both myoglobin and hemoglobin leads to the formation of nitric oxide and the ferric heme derivative. This provides a mechanism for oxygen-dependent release of nitric oxide from GSNO by heme proteins and represents a potential physiological mechanism of S-nitrosothiol metabolism.

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