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THE BIOCHEMISTRY AND PHYSIOLOGY OF S-NITROSOTHIOLS

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■ **Abstract** S-nitrosothiols are biological metabolites of nitric oxide. It has often been suggested that they represent a more stable metabolite of nitric oxide that can either be stored, or transported, although the evidence for this is sparse. There are many unanswered questions concerning how S-nitrosothiols are formed, how they are metabolized and how they elicit biological responses. These questions are highlighted by the fact that the known chemistry of nitric oxide, thiols, and S-nitrosothiols cannot serve to explain their proposed biological activities. This review attempts to highlight the gulf between our chemical understanding of S-nitrosothiols and the proposed biological activities of these compounds with respect to guanylyl cyclase–independent nitric oxide bioactivity and also the control of vascular tone.

INTRODUCTION

S-nitrosothiols are thio-esters of nitrite with the generic structure R-S-N=O (1) and are direct analogs of the nitrite esters of alcohols—the O-nitroso compounds or organic nitrites. These latter compounds have been used for many years as acute vasorelaxing agents. Perhaps the best known of such compounds is amyl nitrite, originally used by Thomas Lauder Brunton in his famous experiments to relieve angina pectoris. N-nitroso amines have been studied for many years in vivo because of their carcinogenic activities. It was not until 1980 that S-nitrosothiols were added to the list of biologically active nitrite esters.

The first indications that S-nitrosothiols may be involved in biological processes came from investigations of guanylyl cyclase activation by O-nitroso and N-nitroso compounds and by metallonitrosyls. Ignarro et al. (2) first postulated a role for S-nitrosothiols in the thiol-dependent activation of guanylyl cyclase by nitrosoguanidine and sodium nitroprusside (3, 4). With the discovery of nitric oxide as a physiological mediator of vascular tone (and many other processes), the potential for the in vivo formation of S-nitrosothiols arose. S-nitrosothiols have been detected in vivo at low concentrations (5–7).

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There is now a large body of literature that implicates S-nitrosothiols as intermediates in nitric oxide–dependent and guanylyl cyclase–independent signaling processes. Reactive protein thiols are becoming regarded as a major intracellular target of nitric oxide. Although there are reports that some enzymes can potentially influence S-nitrosothiol metabolism, the majority of the cyclic guanosine monophosphate (cGMP)-independent effects of nitric oxide are attributed to nonenzymatic chemistry. However, there is a severe mismatch between the known biological chemistry of nitric oxide, thiols and S-nitrosothiols, and the reported effects of nitric oxide in thiol-associated signaling processes. To reconcile the chemical and biological aspects of the NO/thiol interaction, it is necessary to understand how S-nitrosothiols are formed in vivo, how they are metabolized, and how S-nitrosothiol-dependent signaling pathways are controlled.

CHEMISTRY

S-nitrosothiols have the generic structure R-S-N=O. This is not to say that they are adducts of nitric oxide and thiols, or that they represent a bound form of nitric oxide. Both of these statements, often read in the literature, ignore the complexities of S-nitrosothiol chemistry in order to provide a simple mechanism whereby nitric oxide can be stabilized and transported. It is often stated that thiols represent a target for nitric oxide, but the chemical reactivity of nitric oxide indicates that this is not the case. There is also still the myth that S-nitrosothiols can be formed from a simple reversible association of nitric oxide and thiol. In fact the direct reaction between nitric oxide and thiols is a very slow oxidation that yields thiol disulfide and nitroxyl anion (8, 9). The mechanisms that release nitric oxide from S-nitrosothiols are also unclear. S-nitrosothiols do not spontaneously release nitric oxide, although under some circumstances they can decompose or be metabolized to form nitric oxide. This distinction is crucial for experiments in which S-nitrosothiols are used in cell culture, in which formation of nitric oxide is often assumed but not measured.

S-nitrosothiols can be easily synthesized in the laboratory from the reaction between thiols and nitrous acid (Equation 1). This route of synthesis does not occur at physiological pH, and could only be relevant in regions of extreme acidity ($pH < \sim 3$). In the laboratory, this method of synthesis is suitable for low-molecular-weight thiols, such as glutathione and cysteine, but is unsuitable for proteins because of acid denaturation and also because non-thiol protein functional groups, such as amines, alcohols and aromatics, are susceptible to modification by acidified nitrite. For the synthesis of protein S-nitrosothiols, the most often used method involves the spontaneous transfer of the nitroso group from a low-molecular-weight S-nitrosothiol (such as S-nitrosocysteine) to the protein thiol (10).

$$HNO_2 + RSH \longrightarrow RSNO + H_2O \qquad 1.$$

It should be noted, as mentioned above, that the direct reaction between nitric oxide and thiols does not yield an S-nitrosothiol as a product but generates thiol disulfide (Equation 2) (8). In the presence of oxygen, nitric oxide is oxidized to dinitrogen trioxide, which is a good nitrosating agent (Equation 3). Whether this is a viable mechanism for S-nitrosothiol formation in vivo depends largely on whether nitric oxide reacts with oxygen in vivo. This question is still under some debate because the reaction shown in Equation 3 is too slow to explain the rapid consumption of nitric oxide (by unknown mechanisms) in vivo. It can be calculated that less than 0.05% of nitric oxide forms N_2O_3 (assuming a 100 nM steady state nitric oxide concentration, an oxygen concentration of 50 μM, and 10s half life for nitric oxide). The nitric oxide/oxygen reaction can be accelerated in membranes due to favorable partition of both reactants (11), and it is possible that membranes can enhance the yield of thiol nitrosation. This effect may be particularly relevant in membrane-rich environments such as mitochondria (12).

$$\mathrm{NO} + 2\mathrm{RSH} \longrightarrow \mathrm{RSSR} + 2\mathrm{NO}^- + \mathrm{H}^+ \qquad 2.$$

$$2\mathrm{NO} + \frac{1}{2}\mathrm{O}_2 \longrightarrow \mathrm{N}_2\mathrm{O}_3 + \mathrm{RSH} \longrightarrow \mathrm{RSNO} + 2\mathrm{NO}_2^- + 2\mathrm{H}^+ \qquad 3.$$

STABILITY AND BIOLOGICAL CHEMISTRY

As mentioned above, biologically relevant S-nitrosothiols are stable molecules. The bond between the sulfur and nitrogen is a stable covalent bond that is slightly polar (the sulfur is usually more negative than the nitrogen) (13) and is not particularly susceptible to homolysis (i.e., bond breakage to form radicals) unless irradiated with strong, direct light (14, 15). The reason for the observed instability of S-nitrosothiols in solution is that these molecules are susceptible to catalytic decomposition by copper and iron ions that contaminate all buffer solutions (16, 17). For this reason, the half-life of an S-nitrosothiol is directly related to the degree of metal ion contamination and is therefore very variable. In addition, the presence of reducing agents, such as thiols and ascorbate, can enhance metal-ion-dependent decay (18, 19). Metal-ion-dependent decomposition results in the formation of nitric oxide, thiols, and thiol disulfides (20). It is this mechanism that has given rise to the idea that these compounds are nitric oxide donors. However, copper and iron are actively sequestered, in vivo, and may not be available for this reaction. In addition, S-nitrosoglutathione has a built-in safeguard against this route of decay, as oxidized glutathione (GSSG) is a copper chelator that will prevent copper-dependent GSNO decomposition (21). Interestingly, it is the γ-glutamyl carboxylates of GSSG that bind copper, and this protective mechanism is lost through the action of γ-glutamyltranspeptidase (22). Active metabolism of S-nitrosothiols has been observed in a number of systems. Selective chelator studies

have suggested that copper-containing cell-surface proteins exist that can catalyze the degradation of RSNO and consequently enhance its nitric oxide-mimetic activity (22–25). As of yet, such proteins have not been isolated or characterized. Recently, a study identified glutathione-dependent formaldehyde dehydrogenase that catalyzes the NADH-dependent reduction of GSNO to GSSG and ammonia. This study highlights the fact that end-product metabolites of nitric oxide are formed through reduction as well as oxidation (25a). Our studies indicate that bovine aortic endothelial cells metabolize GSNO by a copper chelator-independent mechanism (26). In this study we observed that GSNO metabolism has an absolute requirement for extracellular cysteine but is not saturable. Active metabolism of GSNO has also been observed in neutrophils (27).

Nitrite formation from RSNO is often used as an indication that nitric oxide was released during metabolism (25, 28). This idea comes from the fact that nitric oxide is primarily oxidized to nitrite in oxygenated solution (29). However, it should be realized that nitrite may be formed from RSNO without the intermediate formation of nitric oxide (30) and consequently nitrite is not diagnostic of nitric oxide formation.

Other major biological components that affect the stability of S-nitrosothiols are thiols (31). Two possible mechanisms exist whereby thiols cause the decomposition of S-nitrosothiols. The first is transnitrosation, in which the nitroso group is transferred from an S-nitrosothiol to a thiol (Equation 4).

$$\mathrm{RSNO} + \mathrm{R'SH} \rightleftharpoons \mathrm{R'SNO} + \mathrm{RSH} \qquad 4.$$

The products of this reaction are also an S-nitrosothiol and a thiol, and the reaction is reversible (32). The kinetics of these reactions conforms well to second-order reversible kinetics and generally has rate constants of 1–100 M^{-1} s^{-1} and equilibrium constants close to unity (32, 33). This reaction is important because it represents a mechanism whereby S-nitrosothiol can posttranslationally modify protein cysteinyl residues. Such modification may lead to alterations in activity/structure that can mediate cellular events. S-nitrosothiols are often referred to as NO^+ donors, owing to their ability to modify thiols and other nucleophiles by transnitrosation. In some cases this has been misinterpreted to imply that NO^+ is released as a free entity and that the chemistry and biochemistry of NO^+ is similar to that of S-nitrosothiols (34, 35). However, due to the rapid hydrolysis of NO^+ to nitrite at physiological pH, the lifetime of this cation is about 3×10^{-10} s (36). Peroxidase systems have been shown to oxidize nitric oxide to generate nitrite (37), but the presumed intermediate NO^+ could not be captured as an S-nitrosothiol (R. Glover, R. P. Mason, N. Hogg, unpublished observations). As free NO^+ plays no role in the biochemistry of S-nitrosothiols it is better if this terminology is avoided.

The second mechanism whereby thiols can cause the decay of S-nitrosothiols is S-thiolation (Equation 5). This reaction generates a thiol disulfide and nitroxyl anion (NO^-) (9, 30, 38). Unlike NO^+, NO^- has a significant lifetime that is shortened by the presence of oxygen and thiols and can be regarded as an important biochemical entity. As shown in Equation 5, it is the thiol moiety rather than the

nitroso group that modifies the protein thiol to form a mixed disulfide. The effects on protein activity of S-thiolation and S-nitrosation may be identical, and both modifications should be reversed by reducing agents (such as dithiothreitol). Consequently, S-thiolation and S-nitrosation reactions are indistinguishable based on these criteria. Dithiocarbamates also decompose S-nitrosothiols (39). The use of such compounds as nitric oxide probes cannot distinguish between nitric oxide and S-nitrosothiols.

$$RSNO + R'SH \longrightarrow RSSR' + NO^- + H^+ \qquad 5.$$

A number of groups have reported the decomposition of S-nitrosothiols by superoxide (40–42). The stoichiometry of this reaction was calculated to be 2 superoxides per every S-nitrosothiol decayed. This reaction represents a one-electron reduction of the S-nitrosothiol to form GSH and nitric oxide, the latter of which is immediately scavenged by superoxide to form peroxynitrite (40). Superoxide formation may therefore inhibit the nitric oxide-mimetic properties of S-nitrosothiols. In addition superoxide dismutase enhances GSH-dependent GSNO decomposition to form nitric oxide (43).

S-NITROSOTHIOLS AS MODULATORS OF ENZYME ACTIVITY

As mentioned above, S-nitrosothiols can modify protein thiols by either S-nitrosation or S-thiolation, thereby altering enzyme activity. There are many examples of S-nitrosation in the literature: clotting factor XIII (44), creatine kinase (13, 45), several members of the caspase family of proteases (46), and papain (47). In most cases, however, the protein S-nitrosothiol was not measured, so it is possible that S-thiolation was occurring. GSNO S-thiolates creatine kinase (13), which results in enzyme inhibition. Interestingly, S-nitroso-N-acetyl penicillamine (SNAP) only S-nitrosated this protein (13). S-thiolation of neurogranin by GSNO results in four glutathione moieties per protein, indicating that all four reactive cysteines are modified (48). Although nitric oxide appears to inactivate papain by the formation of an S-nitrosothiol at cysteine 25 (47), incubation of this protein with S-nitrosothiols (GSNO, SNAP, and others), results in S-thiolation of this residue (49). Cellular S-thiolation occurs upon exposure of cells to nitric oxide (50), and S-glutathionylation of glyceraldehydes-3-phosphate dehydrogenase is induced by nitric oxide (51). These examples serve to illustrate that nitric oxide and S-nitrosothiols can modify thiol-dependent processes by a number of different mechanisms.

The observation that a purified protein can be inactivated by incubation with an S-nitrosothiol does little more than identify that the protein contains a cysteinyl residue that is important for enzyme function. Although a consensus motif for the propensity of a cysteine to undergo S-nitrosation has been discussed (52), there are no data to suggest that such a sequence exists. It is likely, as discussed by

Stamler et al. (52), that thiol pKa plays a crucial role in determining the kinetics of transnitrosation, so the local electrostatic- and hydrogen-binding interactions may be important factors. Ascenzi et al. (53) have suggested that a consensus sequence may be an inappropriate concept because the thiol pKa can be altered by amino acid residues at a great distance from the cysteine residue in the primary sequence, but at close proximity in space due to the protein fold. It also should be realized that transnitrosation is a reversible reaction (Equation 4) and that the kinetics of both the forward and reverse reactions needs to be assessed before any judgment of the stability of a particular modification in the intracellular environment can be made. Studies on the transnitrosation reaction, either between low-molecular-weight compounds, or between a low-molecular-weight S-nitrosothiol and a protein thiol, indicate that these reactions are slow ($k = 1–100\ M^{-1}\ s^{-1}$) and reversible, with equilibrium constants close to unity. In the context of a cell, wherein the concentration of glutathione is far higher than any individual protein thiol, a protein with a transnitrosation equilibrium of 1 could never be stably or extensively modified. The kinetic and thermodynamic behavior of transnitrosation argues against a role for this reaction in the control of enzyme activity over short-time scales (i.e., in signaling) and perhaps suggests that the biological manifestations of this chemistry are a stress response to high and prolonged nitric oxide exposure. However, the kinetic limitation could be overcome by catalysis (although no enzymes have yet been discovered) and the thermodynamic issue could be simply due to the fact that our database is not large enough. There are relatively few complete studies of protein transnitrosation, and it is possible that specific protein environments strongly favor S-nitrosation. Understanding the structural basis of such stability will greatly enhance our understanding of this mode of protein modification.

S-NITROSOTHIOLS AND SIGNAL TRANSDUCTION

Signal transduction mechanisms involving S-nitrosothiols and protein S-nitrosation have become an increasing area of interest. Such mechanisms potentially represent guanylyl cyclase-independent routs of nitric oxide bioactivity. One protein that has been studied in this regard is $p21^{ras}$, which can be S-nitrosated in vitro at cysteine 118 (54, 55). Such a modification induces guanidine nucleotide exchange, and it is hypothesized that downstream effects of $p21^{ras}$ activation may be modulated by S-nitrosation in cells as SNAP-mediated MAP kinase activity was inhibited by overexpression of $p21^{ras}C118S$, in which the active cysteine was mutated to a serine residue to prevent S-nitrosation. Although this mutation would also prevent S-thiolation reactions, this study demonstrates the presence of an NO-sensitive thiol in a signaling cascade. S-nitrosocysteine (SNC) and GSNO increase palmitate turnover on the oncogeneic *H-Ras* in NIH3T3 cells (56). This observation was specific for the L-isomer of SNC, which suggests a direct action of the RSNO.

The *c-Myb* transcription factor also contains a cysteine residue (Cys 130) that appears essential for DNA binding. Incubation of this protein with GSNO resulted in inhibition of DNA binding in a concentration-dependent manner in wild type, but not in mutant C130V, *cMyb* (57). In this study S-nitrosation of the cysteine residue was assumed but not examined. In a similar manner, S-nitrosothiols inhibit tumor suppressor *p53* binding to DNA at high concentration, but a lower concentration enhanced DNA binding and nuclear accumulation (58). Although SNAP does elicit a monoclonal antibody-sensitive conformation change in *p53*, the precise chemical and conformational nature of *p53* modification is uncertain (58). The mechanism of macrophage apoptotic-induction by S-nitrosoglutathione is clearly complex, involving not only *p53* but also the regulation of mitogen-activated kinases (59). The transcription factor *NF-κB* can also be inhibited by S-nitrosation of the *p50* subunit (60). This report suggested that endotoxin could mediate this process. In addition, the bacterial transcription factor *OxyR* can be activated by the oxidative stress caused by the presence of nitrogen oxides, perhaps through S-nitrosation (61).

A role for S-nitrosation in apoptosis has been explored due to many observations that demonstrate a cGMP-independent inhibition of many apoptotic stimuli by nitric oxide. One explanation for this is that nitric oxide is a potent antioxidant free-radical scavenger (62) and may suppress apoptosis by removing pro-oxidant molecules. An alternative hypothesis has been developed based on the observation that S-nitrosation of the caspase family cysteine-aspartate proteases can lead to their inhibition (46). Dimmeler et al. have favored S-nitrosation of caspases upstream from caspase-3 processing, such that pro-caspase-3 is never activated in response to Fas activation (63). On the other hand, Stamler and colleagues have favored a dynamic S-nitrosation and denitrosation of caspase-3 controlled by Fas ligand (64). SNC, but not SNAP, inhibited phospholipase A2 activity in PC12 cells, suggesting an interplay between nitric oxide and arachidonate signaling mechanisms (65). The mechanism for this effect is unclear but did not involve guanylyl cyclase activation.

Many cellular signals are mediated through, or controlled by, the activity of ion channels. Many such channels have oxidizable thiol groups that may play a role in the control of channel activity in vivo. The presence of such thiols suggests that regulation by S-nitrosation may also occur. For example, L-type calcium channels are inhibited by nitric oxide (through a guanylyl cyclase-dependent mechanism) but stimulated by S-nitrosothiols (66). Other investigators have reported direct inhibition of L-type calcium channels by S-nitrosothiols (67). Similar observations have been made with the NMDA receptor (68) where differential activities of nitric oxide and S-nitrosothiols were observed.

In addition to ion channels, it is possible that membrane transport proteins can also be affected by thiol modification. GSNO and GSSG have been reported to stimulate L-arginine transport in human platelets (69), providing another potential mechanism, in addition to nitric oxide release, whereby S-nitrosothiols could affect platelet aggregation.

The interaction of nitric oxide with iron-sulfur clusters to generate dinitrosyl-iron-dithiol complexes represents an additional mechanism by which nitric oxide can act through a guanylyl cyclase-independent route. For example, nitric oxide activates SoxR, an oxidant-sensitive transcription factor in *E. coli*, by iron nitrosylation (70). It has been suggested that an interplay between S-nitrosothiols and dinitrosyl-iron complexes may occur (71), and consequently these two pathways of regulation may not be independent. A possible parallel to this reaction can be seen in the interaction of metallothionein with nitric oxide, which can prevent oxidative effects of high nitric oxide formation (72). In addition, S-nitrosothiols activate iron regulatory protein (IRP)'s binding to the iron regulatory element (73). IRP is synonymous with cytosolic aconitase and is an iron-sulfur protein. Interactions of nitric oxide with metal/thiol centers may represent both a signaling and an antioxidant activity.

The potential for guanylyl cyclase-independent routes of nitric oxide action is vast because of the propensity of functionally important intracellular thiols. As far as we know, chemical processes that are not energy-linked, or enzyme catalyzed, control this activity of nitric oxide. The major question that arises is how such processes can be used as control or signaling mechanisms when left to the anarchy of spontaneous chemistry and in the presence of millimolar concentrations of glutathione. It is unclear how a thiol-modifying agent is formed from nitric oxide, in vivo, and once formed it is unclear how it will react with a specific target to modify enzyme activity. Therefore, with the high intracellular glutathione concentration, the search for these processes becomes more like looking for a specific piece of hay, rather than a needle, in a haystack. Although there are now a plethora of examples of thiol-dependent and guanylyl cyclase-independent mechanisms of nitric oxide activity, the fundamental basis for this activity awaits elucidation.

S-NITROSOTHIOLS, NITRIC OXIDE AND THE BLOOD STREAM

Endothelial-derived nitric oxide has a complex and intricate relationship with both the luminal and intimal sides of the endothelium. On the intimal side, the well-established role of nitric oxide as a stimulator of guanylyl cyclase takes precedence; however, additional functions of nitric oxide as a selective modulator of cGMP-independent cellular function are indicated. On the luminal side, the chemistry is dominated by the well-established reactions between hemoglobin and nitric oxide, yet again, the situation may be significantly more complex. The blood stream has long been recognized as the primary route of excretion of the nitric oxide end products, nitrite and nitrate; however, the role of the blood stream in the destruction and/or recycling of nitric oxide remains controversial. Several key observations need to be considered. (*a*) Plasma contains 28 ± 7 nM S-nitrosothiol, which is largely associated with serum albumin (6). (*b*) Oxygenated hemoglobin (oxyHb) rapidly reacts with nitric oxide to form metHb (the ferric derivative) and

nitrate. This reaction is frequently used by investigators to test for and/or quantify nitric oxide formation. Cell-free hemoglobin is a potent vasoconstrictor. (*c*) Nitric oxide binds to deoxygenated hemoglobin (deoxyHb) to form nitrosylHb, which is extremely stable but does oxidize to metHb (after exposure to oxygen) over several hours. (*d*) Erythrocytes contain measurable levels of S-nitrosohemoglobin (HbSNO, i.e., hemoglobin that contains an S-nitroso modification at the β-93 cysteine residue) (74).

These observations have contributed to the idea that blood is more than a garbage disposal route for nitric oxide, and that nitric oxide–derived components of blood contribute to the maintenance and control of vascular tone. One important piece of evidence supporting this view is that inhaled nitric oxide causes peripheral vessel relaxation. This should not be possible, as nitric oxide should be rapidly consumed by oxyhemoglobin. Although there are several potential mechanisms for this effect, the most likely appears to be that nitric oxide is, at least partially, converted into a form that retains endothelium-derived relaxing factor (EDRF) bioactivity (75). S-nitroso-serum albumin is able to relax isolated vessel preparations, so it is possible that the low levels of nitrosated serum albumin contribute to vascular tone (76). OxyHbSNO is a vasoconstrictor, presumably, due to the scavenging of nitric oxide by the oxygenated heme, as metHbSNO has little effect (7). However, in the presence of glutathione (100 μM), oxyHbSNO becomes a vasodilator (7). It can be assumed that glutathione enhances vasodilation via the formation of GSNO. Under anaerobic conditions, the vasodilation elicited by HbSNO was more sensitive to the presence of glutathione than under aerobic conditions, such that 10 μM glutathione caused a greater vasodilation under anaerobic, as compared to aerobic, conditions (77). The fact that physiological concentrations of glutathione are in the 2–3 mM range seems to make this effect irrelevant, because under these conditions, both oxy- and deoxyHbSNO would be vasodilatory. Based on these observations, a hypothesis concerning the oxygen sensitivity of HbSNO-depenent vasodilation has been formulated (7, 77). Briefly, this hypothesis suggests that HbSNO represents an oxygen-sensitive controller of vascular tone such that upon deoxygenation, the nitroso moiety is transferred to glutathione to form GSNO, which can then diffuse or be exported out of the red cell to elicit vasodilation. In a new twist to this hypothesis, McMahon et al. (78) have suggested that the amount of GSNO/nitric oxide formed from this mechanism would cause fatal hypotension. Consequently only a small fraction (0.1%–1%) of the HbSNO is used for the control of vascular tone on each cycle. The remainder of the nitric oxide is autocaptured by the heme and then recycled to the thiol upon reoxygenation. This hypothesis provides a mechanism whereby a hemoglobin molecule can sense the oxygen tension in its vicinity and, if oxygen levels are low, provide a vasodilatory signal. This hypothesis needs to be assessed based on all the experimental evidence available.

There is a thermodynamic relationship between the modification of the hemoglobin thiol and the oxygen affinity of the heme. Following the usual convention, it can be assumed that hemoglobin exists in two states, one of high oxygen affinity

(the R state) and one of low oxygen affinity (the T state). Thiol modification, by agents such as N-ethylmaleimide, forces the protein into an R state and so left-shifts the oxygen binding curve. If the thiol modification is reversible, as in the case of transnitrosation, then the R to T equilibrium position is linked to the equilibrium constants for transnitrosation. All available evidence suggests that S-nitrosation favors the formation of a high oxygen affinity state of hemoglobin, and therefore that transnitrosation from HbSNO to GSH is thermodynamically favored in the deoxygenated state (78–80). This would allow for GSNO formation in low-oxygen conditions. However, care must be taken with the use of the word favored in this context, as the principle of thermodynamic linkage makes no predictions as to the kinetics of the individual reaction steps. It is the kinetics of transnitrosation, and not the thermodynamics, that will be important in determining the amount of vasodilator released during passage of the erythrocyte through the pre-capillary bed.

Our investigations of the kinetics of S-transnitrosation between glutathione and HbSNO have yielded the following data (79). Transnitrosation between GSNO and oxyHb occurs with a rate constant of 0.13 M^{-1} s^{-1} and an equilibrium constant of 1.3. The rate constant is close to the value of 0.365 M^{-1} s^{-1} obtained by Rossi et al. (81) using different techniques. Measurement of transnitrosation under anaerobic conditions has proved to be difficult. We demonstrated that the reaction between GSNO and deoxyHb did not follow reversible second-order kinetics (79). This both questions the mechanism of this interaction and makes the calculation of any equilibrium constants from our data nonsensical. It is therefore puzzling why McMahon et al. (78) calculated equilibrium constants from our data, derived conclusions from these calculations, and then criticized those conclusions as being thermodynamically impossible.

The reason for the deviation of the reaction between GSNO and deoxyHb from the reversible second-order model is that at least one additional reaction is occurring under these conditions. We have recently characterized this reaction and shown that deoxyHb can directly reduce GSNO to generate metHb and nitric oxide (82). The nitric oxide is then autocaptured by a vacant ferrous heme to give nitrosylHb. This observation provides a mechanism for the conversion of deoxyHbSNO to nitrosylHb in the presence of GSH observed by Stamler and colleagues (77, 78). However, these authors suggest that this reaction is reversible in that nitrosylHb can be converted to HbSNO. In our studies we found no evidence for reversibility. The second-order rate constant for transnitrosation of 0.101 M^{-1} s^{-1} measured by Rossi et al. (81) no doubt measures the rate of this reductive reaction.

A key question that has not yet been adequately answered is whether the rate of transnitrosation between HbSNO and GSH is faster under anaerobic conditions. In the absence of GSH, Bonaventura et al. (80) demonstrated that deoxyHbSNO was stable for six days. This is in contrast to Jia et al. (7), who observed an approximate half-time of four hours. Our own experience suggests that the former study is correct. The only direct observation of the rate of decay deoxyHbSNO in the presence of glutathione was made by Jia et al. (7), who reported that the reaction was too fast to measure (i.e., occurred within 15 s). However, this number was

not compared with a similar experiment in the presence of oxygen. Bonaventura et al. (80) reported that about 25–50% of HbSNO remained after deoxygenation, followed by an oxygen titration; however, it is unclear whether loss of HbSNO occurred under deoxygenated or oxygenated conditions as no control was presented for a similar experiment performed without deoxygenation. Interestingly DPG had no effect on the loss of HbSNO, which suggests that the total time of exposure to GSH is more important that the amount of time spent in the oxygenated or deoxygenated state (80). Our data showing that GSH does not appreciably alter the oxygen affinity of HbSNO (at 50% oxygen saturation) over 5 min (79) suggests that this reaction is fairly slow, though no direct comparisons of this rate with that in the oxygenated state can yet be made.

It is now well established that S-nitrosation of the hemoglobin β-93 cysteine increases oxygen affinity (78–80). This suggests the following: A faster rate of transnitrosation in the deoxygenated state would be an issue only when hemoglobin is almost fully deoxygenated. This is perhaps the case in ischemic tissue, as the S-nitrosated component unloads oxygen only after the unmodified Hb becomes deoxygenated.

The issue of how intraerythrocyte GSNO elicits vasorelaxation is an interesting problem that has not been addressed experimentally. Jia et al. (7) measured the release of RSNO from loaded erythorocytes and showed significant release over a one hour time period. This experiment was performed under oxygenated conditions, and the effects of deoxygenation were not examined. Surprisingly, this same group stated that GSNO is highly reactive in the intraerythrocytic environment and liberates nitric oxide (78). This latter claim is likely correct, not because the presence of reducing agents makes GSNO highly reactive, as stated by McMahon et al. (78), but because GSNO reacts with unliganded ferrous heme to generate nitric oxide, as mentioned above. Whether or not any of this nitric oxide or S-nitrosothiol can ever be released from a circulating erythrocyte to elicit a vascular response remains speculative. A recent study showing that a large proportion of erythrocyte S-nitrosothiol is associated with band III does not explain how nitric oxide bioactivity can escape the erythrocyte, but adds another chemical step in this process with unknown kinetic and thermodynamic parameters (78a).

The interaction of nitric oxide with components of the blood stream is clearly complex. The issue of whether the blood components contribute to nitric oxide-mediated control of vascular tone, or whether the blood stream is a garbage chute for spent nitric oxide, has not yet been settled. As in most cases, the true situation may lie somewhere in the middle.

CONCLUSIONS

S-nitrosothiols represent biological metabolites of nitric oxide. Their role in vivo is under increasing examination, and their pharmacological uses have not yet been fully explored. The chasm between the known chemical behavior of S-nitrosothiols

and their proposed in vivo role needs to be addressed in order to understand how S-nitrosothiols can mediate cGMP-independent mechanisms of nitric oxide activity. The major intracellular role of S-nitrosothiols may be in response to oxidative stress, in which thiol modifications by oxidants have been shown to regulate many facets of the stress response. Intracellular S-nitrosothiol formation can be viewed as another marker of oxidative stress, in a similar manner to GSSG formation. The fact that oxides of nitrogen can potentially elicit the same signaling pathways as partially reduced oxygen intermediates is perhaps a testament to cellular efficiency. GSNO is an oxidized form of GSH, and it appears as though GSH oxidation is a common intracellular result of oxidant stress and a mediator of the stress response.

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