

Reaction of Nitric Oxide with the Free Sulfhydryl Group of Human Serum Albumin Yields a Sulfenic Acid and Nitrous Oxide[†]

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ABSTRACT: Nitric oxide (NO) generated by diethylamine nonoate (DEA/NO), an NO donor, readily oxidized the free sulfhydryl group of human serum albumin (HSA) as well as the sulfhydryl groups of reduced glutathione (GSH) and dithiothreitol (DTT) at pH 7.4 and 37 °C. Under anaerobic conditions, the major products of the oxidation of HSA thiol by NO were the sulfenic acid (RSOH) of HSA and nitrous oxide (N₂O). The stoichiometry for this reaction, viz., 1 mol of HSA sulfhydryl oxidized to 1 mol of N₂O produced, is consistent with a net two-electron oxidation of the protein thiol to a sulfenic acid. The sulfenic acid product of HSA was shown to react with dimedone and GSH, two known reactions of sulfenic acids. In contrast, anaerobic oxidation of GSH and DTT by NO gave a stoichiometry close to the expected ratio of 2:1 (sulfhydryl oxidized to N₂O produced) for the oxidation of these thiols to their disulfides and N₂O. Under aerobic conditions, significant fractions of the sulfhydryl groups of HSA, GSH, and DTT were oxidized to their respective thionitrites, presumably by N₂O₃. Thionitrite formation was not observed in the absence of oxygen. The production of HSA-sulfenic acid by NO, as well as by other oxidizing agents such as H₂O₂ and peroxynitrite, followed by its reaction with circulating GSH or L-Cys may account for the mixed disulfides of HSA observed in plasma.

Recent reports suggest that nitric oxide (NO)¹ reacts directly with sulfhydryl-containing substances to yield S-nitroso compounds (Girard & Potier, 1993; Park et al., 1993). Indeed, gaseous NO added to oxygen-free solutions of thiols has been used for the preparation of their S-nitroso derivatives (Mellion et al., 1983; Stamler et al., 1992a). However, for electrophilic nitrosation, other oxides of nitrogen, e.g., HNO₂, N₂O₃, N₂O₄, and alkyl nitrites (Williams, 1988), serve as better sources of the nitrosonium ion (NO⁺). NO itself is not a nitrosating agent at pH 7.4 (Butler & Williams, 1993; Wink et al., 1994).

Under anaerobic conditions, NO reacts directly with low molecular weight thiols at physiologic pH, but the stable end-products of this reaction are a disulfide and nitrous oxide (eq 1; Pryor et al., 1982). In the absence of other thiols,



human serum albumin (HSA), which contains a single free sulfhydryl group (Cys-34; Peters, et al., 1985), should give the homodimeric disulfide. However, when the free thiol of albumin is oxidized by other oxidants, such as H₂O₂ or peroxynitrite, dimers of albumin were not observed (Radi

et al., 1991). The lack of intermolecular disulfide bond formation was attributed to the molecular size of albumin and to steric constraints imposed by neighboring groups surrounding the free thiol group of HSA.

In this report, the end products of the anaerobic reaction of NO with the presumed Cys-34 of HSA are identified and compared with those products resulting from the NO oxidation of reduced glutathione (GSH) and dithiothreitol (DTT). Both gaseous NO and diethylamine nonoate (DEA/NO; Maragos et al., 1991) were used as sources of NO. The NO-oxidized form of HSA was identified as a sulfenic acid, the same product that is formed by oxidation of albumin with H₂O₂ or peroxynitrite (Radi et al., 1991). However, when these reactions were carried out under aerobic conditions, a significant amount of S-nitrosothiol formation was observed with all three of the above thiol-containing substances.

MATERIALS AND METHODS

Materials. HSA and DTT were purchased from Sigma Chemical (St. Louis, MO) and DEA/NO from Cayman Chemical (Ann Arbor, MI). N₂O gas standards and NO gas were from Matheson Gas Products (Chicago, IL). Stock anaerobic solutions of DEA/NO were prepared in 0.1 N KOH and flushed with a stream of Ar. Solutions of NO (2.0 mM) were prepared as previously described (Archer et al., 1995).

Preparation of Thionitrites. Human S-nitrosoalbumin (S-NA) was prepared using acidified nitrite as described by Stamler et al. (1992a) or with isoamyl nitrite. S-NA was prepared using isoamyl nitrite by incubating 0.1 mM DTT-reduced HSA with 2.0 mM isoamyl nitrite in 100 mM potassium phosphate, pH 7.4, for 30 min at 37 °C under anaerobic conditions. Thirty-five percent of the total free thiol of HSA was converted to S-NA. An acidified nitrite

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¹ Abbreviations: DEA/NO, diethylamine nonoate; DTT, dithiothreitol; EDTA, ethylenediamine-N,N,N',N'-tetraacetic acid; GSH, reduced glutathione; HSA, human serum albumin; NO, nitric oxide; N₂O, nitrous oxide; S-NA, S-nitrosoalbumin.

procedure was also used in the preparation of the thionitrites of GSH and DTT (Keaney et al., 1993). The absorption maxima for GSNO and the thionitrite of DTT were 336 ($\epsilon = 902 \text{ M}^{-1} \text{ cm}^{-1}$) and 330 nm ($\epsilon = 990 \text{ M}^{-1} \text{ cm}^{-1}$),² respectively.

Reduction of Albumin. Commercial HSA was reduced with DTT using a modification of a procedure previously described by Katchalski et al. (1975). Solutions of albumin (65 mg/mL) in 20 mM potassium phosphate buffer (pH 6.0), 200 mM KCl, 0.1 mM EDTA, and 10 mM DTT were initially dialyzed against 250 mL of the same solution without albumin for 17 h at 4 °C. The protein was then dialyzed against two changes of a dialysate (1 L) composed of 20 mM potassium phosphate (pH 6.0)/200 mM KCl/0.1 mM EDTA and four changes of the same dialysate, except at pH 7.0. All solutions were deoxygenated with nitrogen and maintained in a nitrogen atmosphere.

Reaction of NO with Thiols. DEA/NO³ was added to 100 mM potassium phosphate buffer (pH 7.4) containing the thiol in a total volume of 1.0 mL in a 22 mL glass septum vial preflushed with Ar to achieve an anaerobic atmosphere. Reaction mixtures with DEA/NO were mixed and then incubated for 30 min at 37 °C without shaking.⁴ Using a gastight syringe, a 0.6 mL aliquot of the headspace was immediately removed and analyzed for N₂O by GC with thermoconductivity detection as previously described (Nagasawa et al., 1990). Parallel incubations were carried out for monitoring the effect of DEA/NO on free sulfhydryl content using Ellman's reagent (Habeeb, 1972).

Protein Analysis. Protein content was determined using bicinchonic acid reagent with HSA as the standard (Smith et al., 1985).

RESULTS

Preparation of Reduced HSA. Using Ellman's reagent, we found that only 20% of commercial HSA was in the reduced form, while the remaining 80% was presumed to exist as mixed disulfides (King, 1961). The mixed disulfides were reduced by treatment with DTT at pH 6.0 using conditions described by Katchalski et al. (1975). Approximately 95–100% of the presumed Cys-34 was converted to the free sulfhydryl form after 17 h of treatment with DTT at 4 °C. When DTT treatment was carried out at pH 7.0, some of the intramolecular disulfide bridges as well as the mixed disulfides were reduced (data not shown).

S-Nitrosation of HSA. Our initial attempts to prepare S-NA were carried out by simply exposing a deoxygenated solution of HSA contained in dialysis tubing to NO gas which was bubbled into the dialysate for 15 min, as previously described by Stamler et al. (1992a). Although the concentration of the free thiol of HSA was significantly reduced by this exposure to NO, the product formed was not a thionitrite, but rather a substance with physicochemical

Table 1: N₂O Production Following the Anaerobic Reaction of GSH, DTT, and HSA with DEA/NO-Derived NO^a

thiol added (nmol)	DEA/NO (1.0 μ mol)	measured SH ^b (nmol)	N ₂ O formed ^b (nmol)	Δ SH/N ₂ O ^c (mol/mol)
none	0	+	<1.0	
DTT	50	–	98.9 \pm 1.1	
DTT	50	+	1.5 \pm 0.4	47.6 \pm 2.6 2.05 \pm 0.11
GSH	102	–	110.7 \pm 2.1	
GSH	102	+	0.6 \pm 0.1	62.6 \pm 3.4 1.76 \pm 0.10
albumin	74	–	71.9 \pm 1.9	
albumin	74	+	2.7 \pm 0.1	69.1 \pm 3.8 1.00 \pm 0.06

^a Experimental conditions were as described under Materials and Methods. The albumin concentration was based on a molecular weight of 65 500. ^b The results are expressed as mean \pm SEM of triplicate samples. ^c The calculated values are given as mean \pm 95% confidence limits.

properties distinctly different from those of an S-nitroso derivative. For example, this NO-modified HSA did not exhibit the characteristic absorption maxima at 320–360 nm for thionitrites, and nitrite was not released by reaction with Hg²⁺ (Saville, 1958).

DTT-reduced HSA was readily converted to S-NA using two different nitrosating agents, namely, acidified-nitrite, i.e., HNO₂ (Stamler et al., 1992a), and isoamyl nitrite. Both S-NA preparations showed an absorption maximum at 336 nm ($\epsilon = 3528 \text{ M}^{-1} \text{ cm}^{-1}$) and produced significant nitrite upon addition of Hg²⁺ (Saville, 1958). With isoamyl nitrite, the amount of HSA thiol that disappeared was shown to equal the amount of S-nitroso adduct formed, the latter based on the Saville assay. From these results, we concluded that exposure of DTT-reduced HSA to nitrous acid or isoamyl nitrite converted its free sulfhydryl group to a nitrosothiol, whereas the product derived from exposure of HSA to NO gas under anaerobic conditions was distinctly different.

NO Oxidation of HSA. Since NO readily oxidizes small molecular weight thiols to their disulfides (Pryor et al., 1982), the possibility was considered that the NO-HSA product was simply a disulfide. In the oxidation of thiols by NO, 2 mol of thiol are oxidized for each mole of N₂O produced (eq 1) so that the ratio of sulfhydryl oxidized to N₂O formed is 2:1. When reduced HSA was treated with DEA/NO-derived NO, the ratio of moles of HSA sulfhydryl oxidized to moles of N₂O produced was approximately 1:1 (Table 1), while similar treatment of DTT and GSH yielded a stoichiometry close to the expected ratio of 2:1. These ratios remained relatively unchanged over a range of GSH, DTT, and HSA concentrations (Figure 1).

The above stoichiometry for the NO reaction with the HSA sulfhydryl group suggests an overall two-electron oxidative process which is consistent with the conversion of a thiol (RSH) to a sulfenic acid (RSOH). Since H₂O₂ also oxidizes the thiol of albumin to a sulfenic acid (Radi et al., 1992) and since both GSH and dimedone are known to react with sulfenic acids to form stable products (Allison, 1976), the effects of GSH and dimedone on the HSA-DEA/NO- and HSA-H₂O₂-products were compared (Table 2). DEA/NO- and H₂O₂-treatment of HSA caused a respective 95 and 84% decrease in its sulfhydryl content. The DEA/NO- and H₂O₂-treated forms of HSA, in turn, were able to oxidize approximately 50% of the added GSH, presumably according

² The ϵ given was determined under acidic conditions (25 °C). Under these conditions, the thionitrite of DTT was stable for at least 10 min, whereas at pH 7.4 (25 °C) this thionitrite gradually decomposed with a half-life of 5.2 min.

³ One mole of DEA/NO decomposes to one mole of diethylamine and two moles of NO in a first-order reaction with a half-life of 2.1 min at pH 7.4 and 37 °C (Maragos et al., 1991). See also footnote 5.

⁴ Shaking the reaction vessels during the incubation period facilitates the diffusion of NO into the headspace; and, therefore, significantly more N₂O production and thiol oxidation was observed *without* shaking.

Table 2: Effect of GSH and Dimedone on the Product Formed from DEA/NO- and H₂O₂-Treated HSA^a

additions	measured RSH (nmol)		
	reduced HSA	DEA/NO-treated HSA	H ₂ O ₂ -treated HSA
none	109.7 ± 0.9	5.6 ± 0.4	18.0 ± 0.4
GSH (100 nmol)	208.5 ± 3.8	46.1 ± 5.3	52.2 ± 2.5
dimedone (200 nmol)	115.8 ± 1.7	4.0 ± 0.2	13.8 ± 0.6
dimedone plus GSH added 30 min later	208.5 ± 4.8	85.9 ± 3.8	78.6 ± 2.0
GSH plus dimedone added 5 min later	203.1 ± 0.6	45.9 ± 2.3	49.4 ± 2.1

^a Initially, HSA (0.1 mM) was incubated with no additions (reduced HSA), 1.0 μmol of DEA/NO (DEA/NO-treated HSA), or 400 nmol of H₂O₂ (H₂O₂-treated HSA) for 20 min at 37 °C under anaerobic conditions in 100 mM potassium phosphate, pH 7.4. After treatment, the headspace of the DEA/NO-treated samples was flushed with nitrogen gas to remove excess NO, whereas 7 units/mL of catalase was added to the H₂O₂-treated samples to remove excess H₂O₂. The mixtures were then incubated for 5 min at 37 °C with GSH, where indicated, and another 30 min with dimedone, where indicated. In the last set of incubations, the order of addition of GSH and dimedone was reversed. The final volume of all reaction mixtures was 1.0 mL.

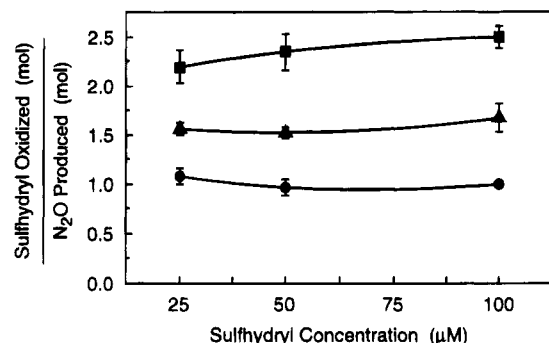
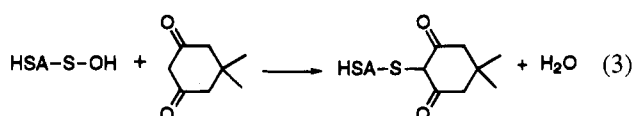
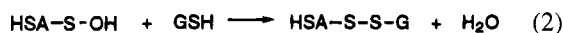


FIGURE 1: Stoichiometry for the production of N₂O versus sulfhydryl oxidized for the oxidation of albumin (●), GSH (▲), and DTT (■) by DEA/NO-derived NO. Experimental details were as described in Table 1. Data are expressed as mean ± 95% confidence intervals.

to eq 2. Dimedone predictably had no effect on the decrease



in sulfhydryl content, but protected the subsequently added GSH from oxidation by DEA/NO- and H₂O₂-treated HSA. Dimedone had no effect when added *after* GSH. On the basis of these results, we conclude that oxidation of the free thiol group of HSA by NO yields a sulfenic acid which is converted to a mixed disulfide by GSH (eq 2) and to a mixed sulfide by dimedone (eq 3).

Oxidation of HSA Thiol by DEA/NO under Aerobic Conditions. The oxidation of HSA, GSH, and DTT by DEA/NO-derived NO was compared using reaction atmospheres of 0:100, 6:94, and 21:78 (air) oxygen/nitrogen in percent (Figures 2 and 3). While all of these thiols were oxidized to some degree by oxygen in the absence of DEA/NO, the sulfhydryl groups of DTT were more susceptible to air oxidation than those of HSA or GSH (Figure 2). The concentration-dependent loss of HSA sulfhydryl was more extensive under anaerobic conditions than in the 6 or 21% oxygen-saturated solutions. As the DEA/NO concentration was increased, oxygen became limiting in incubations with the 6% oxygen saturated solution since the amount of oxygen in solution at 37 °C is only 57 nmol/mL (Lessler & Brierley, 1969) compared to substantially larger amounts of DEA/NO-derived NO. This may explain the observed sharp drop in the free sulfhydryl content of HSA under these conditions.

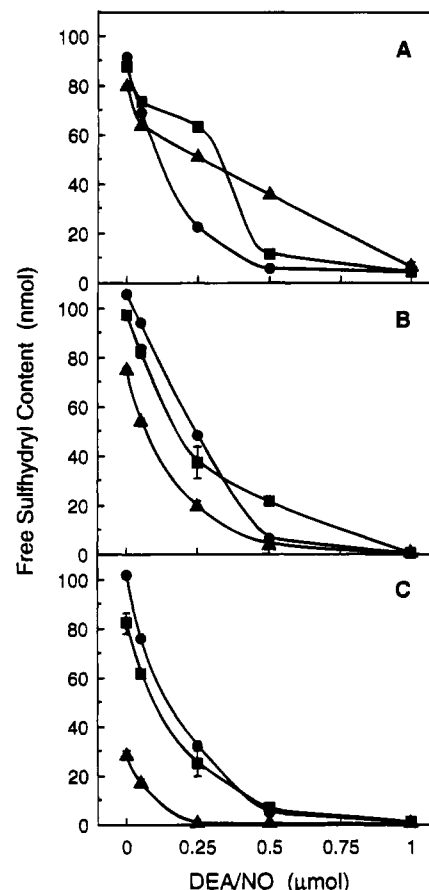


FIGURE 2: DEA/NO-derived NO oxidation of free sulfhydryl of HSA (panel A), GSH (panel B), and DTT (panel C) under anaerobic (●), 6% oxygen (■), and aerobic (▲, air) atmospheres. Experimental conditions were as described in Table 1, except the incubation time was reduced to 20 min and the incubations were carried out in sealed 5 mL Reacti septum vials (Pierce, Rockford, IL). Data are given as means ± SEM.

The NO oxidation of thiols may result in the formation of varying amounts of sulfenic acid, disulfide, and thionitrite. In the oxidation of monothiols by DEA/NO-derived NO, S-nitrosation increased with increasing concentrations of atmospheric oxygen (Figure 3). For each of the oxygen concentrations used, significantly greater amounts of GSNO than S-NA were formed. In the air-saturated reaction mixtures, 66% of the total GSH thiol oxidized was in the form of its thionitrite, while only 21% of the total HSA thiol oxidized was in the form of a thionitrite. Under the same conditions, measurable amounts of thionitrite were also produced with DTT (a dithiol); however, the concentration

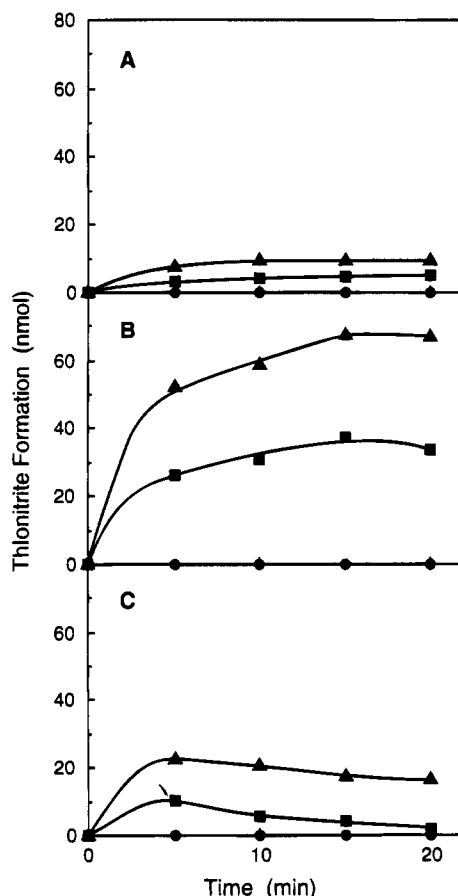


FIGURE 3: Time courses for the S-nitrosation of HSA (panel A), GSH (panel B), and DTT (panel C) by DEA/NO-derived NO under anaerobic (●), 6% oxygen (■), and aerobic (▲, air) atmospheres. Experimental conditions were as described in Table 1, except the DEA/NO concentration was 0.5 mM and the reactions were carried out in sealed septum cuvettes (Spectrocell Corp., Orelana, PA) using a 3 mL reaction volume. The amount of thionitrite formed was determined spectrophotometrically by following the absorption maxima at 336 nm for S-NA and GSNO and at 330 nm for the thionitrite of DTT over time. Representative single 20 min time courses are given for each reaction condition.

of this thionitrite peaked early and then gradually declined over time (Figure 3).

The amounts of thionitrite formed from HSA, GSH, and DTT by exposure to either NO gas or DEA/NO-derived NO under anaerobic conditions were below the detection limits of our assay (Figure 3). However, the presence of even trace amounts of oxygen in these incubation systems resulted in the formation of measurable thionitrite.

DISCUSSION

Our results show that, under anaerobic conditions, NO oxidizes small molecular weight thiols to their corresponding disulfides as previously reported by Pryor et al. (1982) but oxidizes the free sulfhydryl group of HSA (Cys-34) to a sulfenic acid rather than to a disulfide or a thionitrite. Since the nitrogen of NO must first be oxidized to an oxidation state of +3, for example, to N_2O_3 or to another yet unidentified oxide of nitrogen (Wink et al., 1993), for nitrosation, the reaction between NO and a thiol under anaerobic conditions would not be expected to produce an *S*-nitroso substance. On the other hand, when thiols were treated with nitrous acid or isoamyl nitrite or with NO in

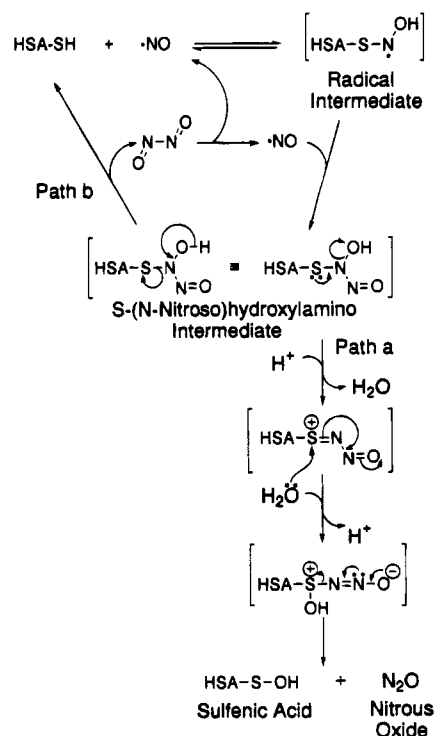
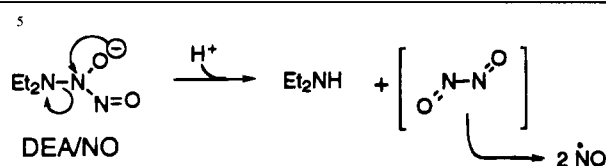


FIGURE 4: Proposed mechanism for the oxidation of the free sulfhydryl group of albumin by NO.

the presence of oxygen, formation of thionitrites was readily demonstrated.

The proposed reaction mechanism for the oxidation of the free sulfhydryl group of HSA by NO is shown in Figure 4. According to this mechanism, 2 mol of NO are required for the oxidation of 1 mol of HSA thiol to give an *S*-(*N*-nitroso)-hydroxylamino intermediate, a net two-electron oxidation of the sulfur atom. This intermediate may then either undergo solvolytic disproportionation to a sulfenic acid and N₂O (path a) or recycle back to a free thiol and NO (path b) to repeat the process. The recycling mechanism is based, in part, on the structural similarities between the postulated *S*-(*N*-nitroso)hydroxylamino intermediate and the nonoate NO donors⁵ (Maragos et al., 1991). Since thionitrite formation was not observed during NO oxidation of thiols under anaerobic conditions, we conclude that a nonsolvolytic decomposition of the *S*-(*N*-nitroso)hydroxylamino intermediate to a thionitrite and nitroxyl (HNO, scheme not presented) does not occur under our experimental conditions.

Due to the electrophilic character of the sulfenyl sulfur, sulfenic acids react with thiols to produce disulfides (Allison, 1976). This accounts for the partial disappearance of the GSH thiol when GSH was added to NO-oxidized HSA (Table 2 and eq 2). Sulfenic acids produced by NO oxidation of small molecular weight thiols, such as GSH and DTT, also react rapidly with a second thiol molecule or another suitably positioned thiol group within the same molecule to produce open chain or cyclic disulfides. In the absence of a neighboring thiol or another nucleophile, the sulfenic acid



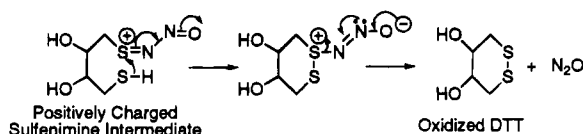
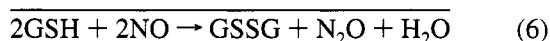
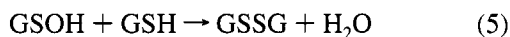


FIGURE 5: Alternative mechanism for the decomposition of the positively charged sulfenimine intermediate formed in the oxidation of DTT by NO.

product from HSA is sufficiently stable to accumulate as observed (Table 2). Oxidation of bovine serum albumin by hydrogen peroxide or by peroxynitrite also leads to the formation of a sulfenic acid (Radi et al., 1991).

Our mechanism for the NO oxidation of thiols to their disulfides (Figure 4 and eq 2) differs from the one proposed by Pryor et al. (1982) in several respects. Their mechanism invoked the dimerization of the initially formed radical intermediate—a statistically unlikely event—and did not involve the formation of a sulfenic acid intermediate. Our evidence in support of a sulfenic acid, albeit indirect, includes the observed stoichiometry for its formation (Table 1 and Figure 1) and its reactivity with GSH and dimedone (Table 2 and eqs 2 & 3).

The overall equations for these reactions are as follows (eqs 6 and 7):



Thus, the products of the oxidation of a thiol by NO under anaerobic conditions can be predicted from the stoichiometry of thiol consumed versus N_2O produced. For example, approximately 2 mol of DTT thiol was oxidized for each mole of N_2O produced, while only 1 mol of HSA thiol was oxidized for each mole of N_2O produced (Table 1 and Figure 1). Similarly, the stoichiometry for the oxidation of GSH, i.e., thiol consumed versus N_2O produced, was 1.76:1 (Table 1), somewhat lower than the predicted 2.0 for disulfide formation. With the relatively low concentration of GSH used in these experiments, it is probable that the final products are a mixture of disulfide and sulfenic acid.

The mechanism for the NO oxidation of DTT and other 1,4-disubstituted dithiols may be somewhat different than that for monothiols. The positively charged sulfenimine intermediate formed from DTT may react with the neighboring sulfhydryl group rather than with water to produce the disulfide and N_2O directly without the intermediacy of the sulfenic acid (Figure 5). Alternatively, once the sulfenic acid is formed, intramolecular disulfide formation should occur rapidly giving the cyclic disulfide. In either case, the expected stoichiometry would be 2:1 as observed (Table 1 and Figure 1).

In the presence of oxygen and DEA/NO, several competing reactions may occur simultaneously. For example, thiols can be oxidized by oxygen (Figure 2) or NO (eqs 1, 6, and 7) or nitrosated by N_2O_3 . In an air atmosphere and in the absence of DEA/NO, DTT was more readily oxidized than HSA or GSH (Figure 2). In the presence of DEA/NO-derived NO, 66% of the oxidized thiol of GSH was in the

form of its thionitrite, whereas only 21% of the oxidized thiol of HSA was thionitrite. While DTT was also nitrosated, its thionitrite was unstable and gradually decomposed over time, presumably to its cyclic disulfide and nitroxyl (Figure 3).

The marked quantitative differences in the products of oxidation of the sulfhydryl groups of HSA, GSH, and DTT following exposure to NO, oxygen, and oxidized forms of NO may be due to a combination of steric factors, influence of neighboring functional groups, and differences in pK_a values of the thiols. The pK_a of Cys-34 in albumin is <5 (Lewis et al., 1980) compared to a pK_a value of 8.72 for the GSH sulfhydryl (Whitesides et al., 1977), while the pK_1 and pK_2 values of 9.2 and 10.1 have been reported for the two sulfhydryl groups of DTT (Reuben & Bruice, 1976).

The oxidation of the sulfhydryl group of HSA by NO, H_2O_2 , and peroxynitrite to a sulfenic acid may explain, in part, the mixed disulfides of HSA found in plasma. The normal concentrations of HSA, GSH, and L-Cys in human plasma are 0.64 mM, 3.4 μM , and 9.0 μM , respectively (Andersson et al., 1993); thus, L-Cys and GSH can readily react with the sulfenic acid of HSA generating these mixed disulfides in human plasma (Peters, 1985).

S-NA is considered to be a carrier form of NO (Stamler et al., 1992b), since it possesses the pharmacologic properties of NO (Keaney et al., 1993). In the presence of oxygen, NO is oxidized to a nitrosating species that nitrosates a substantial fraction of the HSA thiol to a thionitrite. While the concentrations of NO in solution in these studies are significantly higher than those found *in vivo*, the same reactions could still occur *in vivo*, but at a slower rate.

In summary, the oxidation of HSA thiol by NO produces primarily a sulfenic acid under anaerobic conditions. S-Nitrosation of the thiol of HSA or GSH by exposure to NO required the presence of oxygen; indeed, under aerobic conditions a substantial fraction of the oxidized thiol was in the form of a thionitrite.

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