Polynitrosylated proteins: Characterization, bioactivity, and functional consequences

DANIEL I. SIMON*, MARK E. MULLINS†, LI JIA‡, BEN GASTON§, DAVID J. SINGEL¶, AND JONATHAN S. STAMLERT†

*Department of Medicine, Cardiovascular Division, Brigham and Women's Hospital, Boston, MA 02115; †Department of Chemistry, Harvard University, Cambridge, MA 02138; ‡Department of Medicine, Pulmonary and Cardiovascular Divisions, Duke University Medical Center, Durham, NC 27710; ¶Department of Pediatrics, U.S. Naval Hospital, San Diego, CA 92134; and †Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717

Communicated by William Klemperer, Harvard University, Cambridge, MA, January 16, 1996 (received for review January 2, 1996)

ABSTRACT Chemical modification of proteins is a common theme in their regulation. Nitrosylation of protein sulfhydryl groups has been shown to confer nitric oxide (NO)-like biological activities and to regulate protein functions. Several other nucleophilic side chains—including those with hydroxyls, amines, and aromatic carbons—are also potentially susceptible to nitrosative attack. Therefore, we examined the reactivity and functional consequences of nitrosylation at a variety of nucleophilic centers in biological molecules. Chemical analysis and spectroscopic studies show that nitrosation reactions are sustained at sulfur, oxygen, nitrogen, and aromatic carbon centers, with thiols being the most reactive functionality. The exemplary protein, BSA, in the presence of a 1-, 20-, 100-, or 200-fold excess of nitrosating equivalents removes 0.6 ± 0.2, 3.2 ± 0.4, 18 ± 4, and 38 ± 10, respectively, moles of NO equivalents per mole of BSA from the reaction medium; spectroscopic evidence shows the proportionate formation of a polynitrosylated protein. Analogous reaction of tissue-type plasminogen activator yields comparable NO/protein stoichiometries. Disruption of protein tertiary structure by reduction results in the preferential nitrosylation of up to 20 thus-exposed thiol groups. The polynitrosylated proteins exhibit antiplatelet and vasodilator activity that increases with the degree of nitrosation, but S-nitroso derivatives show the greatest NO-related bioactivity. Studies on enzymatic activity of tissue-type plasminogen activator show that polynitrosylation may lead to attenuated function. Moreover, the reactivity of tyrosine residues in proteins raises the possibility that NO could disrupt processes regulated by phosphorylation. Polynitrosylated proteins were found in reaction mixtures containing interferon-γ/lipopolysaccharide-stimulated macrophages and in tracheal secretions of subjects treated with NO gas, thus suggesting their physiological relevance. In conclusion, multiple sites on proteins are susceptible to attack by nitrogen oxides. Thiol groups are preferentially modified, supporting the notion that S-nitrosylation can serve to regulate protein function. Nitrosation reactions sustained at additional nucleophilic centers may have (patho)physiological significance and suggest a facile route by which abundant NO bioactivity can be delivered to a biological system, with specificity dictated by protein substrate.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Nitr oxide (NO) reacts in biological systems with oxygen (O₂), superoxide (O₂⁻), and transition metals to form a variety of nitrogen oxides: NO, peroxy nitrite (ONO⁻), and metal–NO adducts, respectively (1). These species can effect nitrosative reactions at responsive nucleophilic centers (2). Nitrosylation of sulfhydryl centers has been shown to confer NO-like biological activities and/or to regulate protein functions in numerous systems, including low-molecular-weight thiols, such as cysteine (3); plasma proteins, such as albumin (BSA) (4); enzymes, including tissue-type plasminogen activator (t-PA) (5), cathepsin B (4), and glycer aldehyde-3-phosphate dehydrogenase (6); ion channels, such as the charybdotoxin-sensitive potassium channels of vascular smooth muscle (7) and the N-methyl-D-aspartic acid subtype of glutamate receptors (8); G proteins, such as p21ras (9); and nuclear proteins, such as O6-methylguanine-DNA-methyltransferase (10).

Nitrosation of simple alcohols and amines (i.e., oxygen and nitrogen centers, respectively), as well as carbon centers, is well documented (11) and suggests that nitrosation of amino acids with hydroxyl, amino, and other functional groups might be possible and perhaps biologically relevant. For example, under oxidant stress—such as the upregulation of the cytokine-inducible form of NO synthase (12)—thiol pool depletion might be predicted to render multiple functional groups on proteins susceptible to nitrosative attack, and thus, through polynitrosylation, to alter protein function. Therefore, we examined the relative reactivity and the biological consequences of nitrosation at various nucleophilic centers on amino acids and proteins. Using the exemplary proteins BSA and t-PA, large proteins rich in potential nitrosation targets, we find that multiple sites, not limited to thiols, sustain nitrosylation; thiols are, however, the preferred target of nitrosative chemistry. The polynitrosated derivatives of the proteins exhibit some alterations in their intrinsic function but acquire NO-like biological activities, which are most potent for nitrosated thiols.

MATERIALS AND METHODS

Na[15]NO₂ was obtained from MSD Isotopes. L-Boc-Tyr(Et)-OH was purchased from Bachem. NO gas was obtained from Matheson and used without further purification. Human fibri nogen (grade L) was purchased from Enzyme Research Laboratories (South Bend, IN). t-PA was kindly provided by Genentech. Recombinant human plasminogen activator inhibitor 1 (PAI-1) (predominantly active) was kindly provided by Thomas Reilly (DuPont–Merck).

Synthesis of Nitroso Proteins. To investigate the reaction(s) between NO, and nucleophilic centers of proteins, we examined two thiol-containing proteins of different function: BSA, the most abundant plasma protein, and t-PA, an endothelium-derived serine protease. Three methods were used to synthesize nitroso proteins. In the first method, BSA (4.4 mg/ml) and t-PA (4.8 mg/ml, previously dialyzed with 0.1 M HCl to remove l-arginine) were nitrosated by exposure to NaNO₂ in 0.5 M HCl (acidified NO₂) for 15 min at 37°C, as described in

Abbreviations: t-PA, tissue-type plasminogen activator; PAI-1, plas minogen activator inhibitor 1; NO₃, nitrogen oxides.

†To whom reprint requests should be addressed at: Duke University Medical Center, MSRB Room 221, Durham, NC 27710.

‡The covalent attachment of the NO group to sulfhydryl residues in proteins is defined as S-nitrosylation. General NO attachment to nucleophilic centers is referred to as nitrosation.
detail (4). Nitrite concentration varied from equimolar to 1000-fold excess over protein concentration. Solutions were then neutralized to pH 7.4 by the addition of 1.0 M NaOH and Tris-buffered saline (TBS). For some bioassays, nitrosoproteins were desalted of excess nitrite. Native BSA and t-PA were also subjected to acidification in 0.5 M HCl and then neutralized (in the absence of nitrite) to obtain appropriate controls for the functional assays described below.

Protein nitrosylation was also achieved in helium-deoxygennated solutions of 100 mM sodium phosphate, pH 7.4, by exposing the protein solution in dialysis tubing to NO gas bubbled into the dialysate for 15 min. The proteins were then dialyzed against a large excess of PBS, pH 7.4, to remove excess oxides of nitrogen. Tracheal secretions of patients receiving 40–80 ppm of inhaled NO gas for therapeutic purposes, as recently described (13), were also analyzed for the presence of nitroso proteins. Finally, proteins were nitrosated under physiological conditions by exposure to a mouse peritoneal macrophage cell line (RAW 264.7, ATCC) stimulated to produce NO. RAW cells (3 × 10⁶) were cultured in six-well tissue culture plates, stimulated with 100 ng/ml of lipopolysaccharide and 100 units/ml of interferon-γ, and then incubated overnight in serum-free DMEM containing 3 mg/ml of BSA.

Nitroso production and nitroso protein formation were assessed chemically and spectrophotometrically as described below. To investigate whether additional nucleophilic centers are exposed and capable of undergoing nitrosylation after extensive protein reduction, BSA was treated with 100 mM DTT and then dialyzed exhaustively in 0.1 M HCl to remove excess DTT and maintain BSA in the reduced form. DTT-treated BSA was then nitrosated by exposure to NaNO₂ in 0.1 M HCl.

**Chemical and Spectrophotometric Analyses of Nitroso Proteins.** The formation of (poly)nitroso proteins was confirmed by chemical and spectroscopic analyses. The colorimetric assay involving diazoitation of sulfanilamide and subsequent coupling with N-(1-naphthyl)ethylenediamine was used to determine concentrations of nitrosating equivalents (4). A standard curve with acidified NaNO₂ was constructed to correlate concentration with A₅₈₅. Protein-bound NO groups were then quantified by measuring the attenuation of A₅₈₅ relative to the acidified nitrite standard, in the protein reaction mixture. Protein S-nitrosothiol content was determined by monitoring the increase in A₅₈₅ after the selective displacement of the NO group from S-nitrosothiols by Hg²⁺ (4). UV-visible absorbance spectra of (poly)nitroso proteins were measured with a Gilford Response Spectrometer (CIBA–Corning). For ¹⁵N NMR experiments, 50 μmol of L-tyrosine, L-phenylalanine, L-Boc-Tyr(Et)-OH, L-tryptophan, or the catecholamine noradrenaline was dissolved in 0.5 ml of distilled water, Na¹⁵NO₂ (50–100 μmol) was dissolved in 0.5 ml of 1.0 M HCl and transferred immediately to the aqueous solution with agitation. The solution was capped and allowed to sit at room temperature for 30 min. ¹H₂O was then added, and the ¹⁵N NMR measurements were taken immediately. In the case of nitroso proteins synthesized with Na¹⁵NO₂, measurements were made according to the method of Bonnett and coworkers (14). The spectra were recorded with a Bruker AM 500-MHz spectrometer (Billerica, MA). A deuterium lock was effected with ²H₂O, and the spectra were referenced to a ¹⁵N natural-abundance spectrum of a saturated solution of NaNO² assigned to δ 857 (14). Spectra were recorded at 50.68 MHz.

**Platelet Aggregation.** Venous blood was obtained from volunteers who had not consumed aspirin or other nonsteroidal antiinflammatory drugs for at least 10 days, and the blood was anticoagulated with 13 mM trisodium citrate. Platelet-rich plasma (200 μl), prepared as described (4, 5), was then incubated with 100 μl of nitroso protein for 10 min at 37°C in a PAP-4 aggregometer (BioData, Hatsboro, PA), after which aggregation was induced with 5 μM ADP. For aggregation experiments performed with t-PA, 280 kallikrein inhibitor units/ml of aprotinin was added to inhibit plasmin activity and the generation of fibrinogen degradation products. Aggregation was quantified by measuring the rate or extent of change in light transmittance.

**Blood Vessel Bioassay.** Vessel rings were prepared from descending thoracic aortae of New Zealand White rabbits (3–4 kg) according to a published protocol (4). The rings were suspended in glass chambers containing oxygenated Krebs buffer and were connected to transducers (Grass Instruments, Quincy, MA; model FT03C). Sustained contractions were induced with 1 μM norepinephrine, after which the effects of nitroso proteins were tested.

**Fibrinolytic Assay.** The ability of nitrosylated t-PA to cleave fibrin was examined by use of the fibrin plate method as described by Astrup and Mullertz (15) with modification (16). Grade L fibrinogen containing sufficient plasminogen for plasmin generation by the plasminogen activator, t-PA, was used. To a uniform fibrin layer, 20-μl samples containing varying concentrations of t-PA or nitroso-t-PA (1.0–100 nM) in TBS (pH 7.4) were then carefully applied and allowed to incubate at 37°C. After 18 h, the zone of lysis (mm²) was determined from the product of two perpendicular diameters measured with calipers to the nearest 1 mm and then plotted as a function of t-PA or nitroso-t-PA concentration. Samples containing 10 nM t-PA or nitroso-t-PA were also incubated with 100 nM active PAI-1 for 30 min before application to the fibrin layer.

**RESULTS**

**Chemical and Spectroscopic Analyses of Polynitrosylated Proteins.** The exemplary proteins BSA and t-PA undergo polynitrosylation in acidified NaNO₂. Incubation of BSA with 1-, 20-, 100-, and 200-fold excess concentrations of (acidified) NO₂ resulted in the reaction of 0.6 ± 0.2 (mean ± SEM), 3.2 ± 0.4, 18 ± 4, and 38 ± 10 mol of nitrosating (NO⁺) equivalents per mol of BSA, respectively (Fig. 1). Similarly, incubation of t-PA with 1-, 20-, 100-, and 200-fold excess of NO₂ led to the reaction of 0.8 ± 0.1, 5.2 ± 0.2, 20 ± 2, and 54 ± 8 mol of nitrosating equivalents per mol of t-PA, respectively (Fig. 1). These data reflect a linear decrease of unreacted nitrosating equivalents with increasing NO₂/ protein mole ratios under these conditions.

---

**FIG. 1.** Protein polynitrosylation. BSA and t-PA undergo polynitrosylation after exposure to molar excess acidified nitrite, an exemplary nitrosating medium. Values represent mean ± SD for five experiments.
The reaction of BSA and t-PA with equimolar concentrations of acidified NO\textsubscript{2} resulted in the exclusive formation of an S-nitrosylated product, as judged by the displacement of the NO group upon exposure to Hg\textsuperscript{2+}. Reactions carried out with excess NO\textsubscript{2} are consistent with the modification of functional groups other than thiol: the number of Hg\textsuperscript{2+}-displaceable products observed under these conditions is at most one, consistent with the presence of a single free sulfhydryl in these proteins. With increasing NO\textsubscript{2}, the thiol content was observed to decrease, presumably owing to thiol oxidation, and is clearly far less than the number of nitrosating equivalents consumed by the protein.

Confirmatory evidence that these reactions do indeed affect the modification of proteins was obtained by UV-visible spectroscopy. Treatment of BSA or t-PA with excess acidified NO\textsubscript{2} produces yellow solutions with absorption maxima at 340–350 nm, characteristic of nitrosation products (17, 18). Reaction of t-PA or BSA with 1-, 10-, 100-, and 1000-fold molar excess concentrations of acidified NO\textsubscript{2} resulted in a progressive increase in absorption at 340 nm (Fig. 2A), proportional to the extent of nitrosylation as determined in the chemical assay (linear correlation coefficient > 0.9). In the case of reactions carried out with equal reactant concentrations, an absorption peak at 540 nm, indicative of S-nitrosothiol formation, was also detected (data not shown; see also ref. 4). The half-lives of the synthesized polynitrosated proteins, as determined by the combined Saville and Griess reactions (pH 7.4, 25°C, and a protein concentration of 17 μM), range from ≈24 h for S-nitroso-BSA to 48 h for BSA nitrosated with a 20-fold excess concentration of NO\textsubscript{2} (i.e., reacting with 3.2 ± 0.4 mol of NO\textsubscript{2} per mol of BSA).

Protein polynitrosylation was also achieved by exposure of proteins to NO gas or mouse peritoneal macrophages (RAW cells) stimulated with lipopolysaccharide and interferon-γ, as described in Materials and Methods. Conditioned media from the stimulated RAW cells were found, by chemical assay, to be 82 μM in nitrosating equivalents (NO\textsubscript{2}). Absorption spectra obtained after exposure of BSA to RAW cells exhibited a maximum at 355 nm (A\textsubscript{355} = 0.35 cm\textsuperscript{-1}), similar to that observed upon treatment of BSA with excess NaNO\textsubscript{2}. Exposure of BSA to authentic NO gas also yielded the same absorption maximum (354 nm). Tracheal secretions obtained from two patients breathing NO gas (40–80 ppm; fractional concentration of O\textsubscript{2} > 0.6) were yellow-orange and exhibited an absorption maximum at ≈350 nm. Taken together, these data indicate that polynitrosylation reactions are sustained by proteins under physiological conditions.

To gain some insight into the functional groups of BSA and t-PA that can serve as nucleophilic centers in the formation of polynitrosated proteins, \textsuperscript{15}N NMR experiments, conducted after Bonnet and coworkers (14), were conducted: various amino acids and related compounds were nitrosated by exposure to an equivalent of acidified \textsuperscript{15}N-labeled nitrite. Inasmuch as this procedure effectively duplicates one employed in the (poly)nitrosylation of proteins, the nitrosation products observed by \textsuperscript{15}N NMR give some indication of which groups on the protein are susceptible to nitrosative attack.

Like Bonnet and coworkers (14), we observed nitroamines (at ≈550 ppm) and the thionitrite (at ≈750 ppm) derived from l-cysteine. In addition, in experiments on tyrosine and tryptophan, we observed a signal (≈890 ppm) that we assign to an aryl-nitroso species. This species may result from direct nitrosative attack on carbon centers, or perhaps by rearrangement of an initially formed aryl nitrite. Such a reaction pathway is especially intriguing in the case of tyrosine because of its implications for tyrosine phosphorylation. Indeed, exclusively in experiments on tyrosine and the catecholamine norepinephrine (2-fold excess NO\textsubscript{2}), we observed an additional peak at ≈730 ppm that we tentatively suggest might be the initially formed O-nitrosotyrosine. Finally, it is interesting to note that, although this type of reaction typically leads ultimately to a nitro-derivative product, no \textsuperscript{15}N NMR signals assignable to O-nitrotyrosine (19) were observed under these conditions.

\textsuperscript{15}N NMR spectra of BSA and t-PA were also obtained after treatment with equimolar and excess concentrations of acidified Na\textsubscript{15}NO\textsubscript{2}. The \textsuperscript{15}N NMR spectra of BSA and t-PA nitrosated with one equivalent of acidified nitrite exhibited a peak at 750 ppm, consistent with S-nitrosothiol formation (4). Under conditions of polynitrosylation, additional peaks were observed, which, on the basis of characteristic chemical shifts discussed in the study of amino acids above, suggest NO-group attachment at oxygen and nitrogen centers. The NMR signals suggest an order of NO reactivity of thiol > tyrosine > amine. A representative spectrum of nitrosylated BSA is provided in Fig. 2B, showing a peak at ≈750 ppm typical of an S-nitrosothiol, as well as a peak at 730 ppm that appears when the concentration of nitrosating equivalent exceeds that of free thiol.

The intracellular redox state of the cell is thought to be a major determinant of tertiary structure (20). During protein processing in cells, tertiary structure is often disrupted. To determine if additional nucleophilic centers are exposed and capable of undergoing nitrosylation under such conditions, BSA was treated with DTT, extensively dialyzed, and then nitrosated by exposure to a 25-fold excess concentration of acidified nitrite. Under these conditions, extensive thiol nitrosation was observed; specifically, we ascertained that 19.2 ± 3.1 (mean ± SD) mol of S-NO per mol of BSA was formed.
Moreover, total protein-NO was virtually identical to S-nitrosothiol content. This result markedly contrasts with those obtained with the native protein, which are best interpreted as indicating the nitrosation of a single thiol. BSA contains 35 cysteines that form 17 disulfide bonds with one free thiol group in the native conformation (21). Evidently, about half of the (native) cysteine residues become exposed by the DTT reduction and sustain nitrosation. In aggregate, these results demonstrate that protein thiols are preferentially modified: indeed, the exposure of cysteine residues protects against modification at other sites, a finding consistent with 15N NMR predictions that thiols are the most reactive functional groups.

Bioassays of Polynitrosylated Proteins. Platelets are inhibited by exposure to endothelium-derived relaxing factor (22), NO (23), low-molecular-weight nitrosothiols (24, 25), and protein S-nitrosothiols (26). The ability of polynitrosylated BSA and t-PA to serve as donors of NO bioactivity was assayed by examining the inhibition of ADP-induced platelet aggregation. Polynitrosylated BSA and t-PA exhibited antplatelet activity that increases with degree of nitrosation (Fig. 3A and B). S-nitrosylation was found to confer greater antplatelet activity than polymodification of other groups: polynitrosylated S-NO-BSA (reduced), which contains 19.2 ± 3.1 mol of S-NO per mol of BSA, was a significantly more potent platelet inhibitor (IC50 = 0.2 μM) than BSA that had reacted with 38 nitrosating equivalents at its various receptive functional groups, which include only a single thiol (IC50 = 4.4 μM). Interestingly, polynitrosylated BSA and t-PA possess nearly equipotent platelet inhibitory activity (Fig. 3B), consistent with the roughly equivalent stoichiometries of nitrosation after exposure to 1-, 20-, and 200-fold excess of nitrite.

The response in blood vessels to polynitrosylated proteins mimicked that in platelets. Specifically, the potency of vasorelaxant responses increased with the degree of nitrosation, but the poly(S-nitroso) derivative was by far the most potent compound (Fig. 4).

Enzymatic Function and Protein–Protein Interaction of Polynitrosylated Proteins. The ability of t-PA to bind fibrin and activate plasminogen to plasmin was assessed in the classical fibrin plate assay. As depicted in Fig. 5, polynitrosylation of t-PA results in a progressive attenuation of fibrinolytic activity that is related to the degree of polynitrosylation. The fibrinolytic activity of t-PA nitrosated with equimolar NO+ (i.e., S-nitroso-t-PA) is identical to that of t-PA. Polynitrosylation of t-PA, however, significantly reduces, but does not completely arrest, fibrinolytic activity. The residual activity can be completely inhibited by PAI-1. This observation suggests that polynitrosylated t-PA retains the ability to bind PAI-1.

**DISCUSSION**

This study demonstrates that (i) nitrosation reactions are sustained at various nucleophilic centers of proteins, (ii) nitrosation occurs preferentially at exposed thiol groups, (iii) polynitrosylated proteins exhibit NO-like bioactivity that increases with the degree of nitrosation, (iv) S-nitrosylation confers greater bioactivity than modification of other groups, and (v) polynitrosylation of a protein can, but does not necessarily, attenuate its activity.

Mechanism(s) of Protein Polynitrosylation. The mechanism of nitrosation in vivo remains to be elucidated. Several nitrogen oxides (NOx), including dinitrogen trioxide (N2O3), peroxynitrite (OONO-), and metal-NO complexes (free and related centers in proteins), can support nitrosation reactions (2). Various nitrosation mechanisms, involving different intermediates, could apply, depending on the cell system and ambient conditions. For example, in the gut the low pH is conducive to nitrosation via NO+ (transfer), effectively mimicked in our studies by acidified nitrite. In the airways, NO/O2 reactions [with a nitrosating species presumed to be N2O3 (13, 27)] probably happen, whereas macrophages may marshal

---

**FIG. 3.** Antiplatelet properties of polynitrosylated proteins. Polynitrosylated BSA (A) and t-PA (B) exhibit antplatelet activity that increases with degree of nitrosation affected by exposure to 1-, 20-, and 200-fold molar excess of acidified nitrite; P < 0.05 by ANOVA). Polys-nitrosylation (after protein denaturation with DTT) confers greater antplatelet activity than polymodification of other groups (A) (P < 0.001 by ANOVA). Polynitrosylated t-PA exhibits comparable bioactivity to polynitroso-albumin at 1- and 20-fold molar excess of NOx but is more potent after treatment with the 200-fold excess concentration (B). NaN03 alone has no significant effect on platelet aggregation over the range of concentrations tested. Aggregation is expressed as a normalized value relative to control.

**FIG. 4.** Vasorelaxant properties of polynitrosylated proteins. Polynitrosylated proteins exhibit relaxant activity that increases with the degree of polynitrosylation (affected by exposure to 2-, 20- and 200-fold molar excess acidified nitrite), thus mimicking the responses in platelets (Fig. 3). Polys-nitrosylated proteins engender the most potent vasorelaxant response. Nitroso proteins were desalted of excess nitrite before study.
multiple mechanisms. In this study, the exposure of proteins to acidified NaNO₂, “authentic” NO gas, or cytokine-stimulated mouse peritoneal macrophages resulted in protein polynitrosylation. Regardless of the mechanism(s) of polynitrosylation, it is clear that both exogenous and endogenous nitrosating reagents are effective in nitrosylating various centers in proteins, preferably, but by no means exclusively, protein thiols. To the extent that the 15N NMR signal strengths can be taken as indicating an order of reactivity, our results are in agreement with the findings of Wink and coworkers (28) and Mirza and coworkers (29) working with single amino acids and small peptides, and of Massey and coworkers in related model systems (30), who found relative reactivities thiol > tyrosine > amines.

There is abundant evidence that S- and N-nitrosation reactions are of biological relevance. S-nitrosylation of amino acids, peptides, and proteins has been reported under physiological conditions and appears to have regulatory consequences (2, 4-10, 13). In contrast, N-nitrosation reactions are implicated in mechanisms of cellular injury and mutagenesis. Pathways include the nitrosation of primary amines in DNA and its constituent deoxynucleotides, which leads to deamination, and the formation of nitrosamines, which similarly act as powerful carcinogens (11, 12). Analysis of the fate of nitrite at gastric pH has also revealed interactions with protein amino groups that yield other modified products (17).

Nitrosation of tyrosine requires additional comment. While O-nitrosation of simple alcohols has been reported, nitrosation of phenols ultimately yields the ortho and para ring-substituted derivatives (31); a Fisher–Hepp rearrangement of an initially formed O-nitroso species has been proposed (32). The data reported here are consistent with this scheme. Processes regulated by tyrosine phosphorylation would be perturbed by competitive nitrosation reactions, although the biological relevance of this reaction remains to be demonstrated. Furthermore, it is intriguing to speculate on the bioactivity of O-NO intermediates, recalling that alkyl nitrates possess antiangiial activity. Indeed, polynitrosylated proteins may have therapeutic potential as they offer a facile route by which abundant NO bioactivity can be delivered to a biological system with specificity dictated by protein substrate. It is also somewhat intriguing that nitration of tyrosine (NO₂ substitution) is observed in vivo in settings of oxidative stress (33) that support nitrosative chemistry (12). In fact, nitration of tyrosyl residues of proteins and their oxidation to nitrotyrosine has been previously demonstrated under physiological conditions (17, 28). Thus, this pathway provides an additional mechanism by which nitrotyrosine could be formed in vivo.

**Polynitrosylated Proteins: NO Buffers vs. NO Donors.** Polynitrosylated BSA and t-PA exhibit antiplatelet and vasodilator activities that increase with the degree of nitrosation, indicating that both thiol and nonthiol (i.e., nitrogen and/or oxygen) centers are a source of NO-related bioactivity. However, S-nitrosylation confers greater bioactivity than modification of other groups. Also, in distinct contrast to NO, nitroso proteins are long-lived in physiological media, reflecting the relative lack of reactivity of the protein-bound NO group toward O₂ species. It follows that nucleophilic sites on proteins may be viewed as effective “sinks” that buffer NO responses. One might envision, for example, that a burst of NO synthase activity could charge an intracellular protein in its immediate vicinity, which would then slowly release NO for some physiological function. These proteins are also reasonable candidates for the NO-related storage pool in vascular smooth muscle that is released by photolysis and replenished by nitrosating agents (34). Whether polynitrosylation of proteins also serves as a mechanism of toxicity will likely depend on circumstance. We speculate that low-level polynitrosylation will be well tolerated, even acting to protect the cell from the oxidant effects of NO₃ in much the same way that proteins buffer against oxygen free-radical injury. On the other hand, unregulated high-output production of NO₃, as occurs with lipopolysaccharide/interferon-γ-stimulation of murine macrophages in vitro, can ultimately lead to cell demise, under which circumstance excessive protein modification may be one contributing factor.

**Polynitrosylated Proteins: Modification of Function.** We observed that polynitrosylation is, not surprisingly, capable of modifying protein function. This is exemplified in the attenuation of the serine protease activity of t-PA. At the same time, it is important to emphasize that polynitrosation does not necessitate loss of function. Indeed, attachment of 54 NO groups to t-PA did not quash protease-serpin interaction (i.e., residual fibrinolytic activity was completely inhibitable by PAI-1). The ability of NO groups to modify protein activity will critically depend on both the reactivity of the constituent amino acids and their relative importance in determining protein structure/function.

Thiol groups are preferentially modified by nitrosation. The implications are twofold. First, protein sulphydryl residues as well as the low-molecular-weight thiol pool (13) may serve an important detoxifying role, protecting against untoward reactions at other sites; second, posttranslational modification of proteins for regulatory purposes is unlikely to occur at nucleophilic centers beside thiol. These contentions are supported by the fact that thiol is the major nucleophilic target in proteins involved in NO responses (2). Furthermore, as cysteine residues are also sites for covalent attachment of other regulatory molecules (i.e., prenylation, ADP-ribosylation, and palmitoylation), S-nitrosylation may have implications extending to other signaling pathways. In fact, modulation of thioester-linked long-chain fatty acylation of neuronal proteins by NO has been shown to affect process outgrowth and remodeling (35).

The preferential attachment of NO⁺ to thiol groups assumes additional importance given that tertiary structure is often disrupted during cellular processing. Specific intracellular (36) and extracellular (37) transport systems deliver thiol that can break protein disulfide bridges, thereby exposing potential S-nitrosylation sites. Analogous consideration has been given to the role of protein tertiary structure in determining the extent of phosphorylation (38). Possible regulatory functions for S-nitrosylation of intramolecular sites uncovered during protein degradation merit further consideration.
The authors acknowledge the technical assistance of Hui Ling Li and Hui Xu. D.J.S. is a recipient of National Institutes of Health Clinical Investigator Development Award HL02768. J.S.S. is a few scholar in the biomedical sciences and the recipient of National Institutes of Health Clinical Investigator Development Award HL02582 and Grant HL52529.