S-Nitrosylation of protein thiols is one of the cellular regulatory mechanisms induced by NO. The cysteine protease papain has a critical thiol residue (Cys²⁵). It has been demonstrated that NO or NO donors such as sodium nitroprusside and N-nitrosoaniline derivatives can reversibly inhibit this enzyme by S-NO bond formation in its active site. In this study, a different regulated mechanism of inactivation was reported using S-nitrosothiols as the NO donor. Five S-nitroso compounds, S-nitroso-N-acetyl-L- penicillamine, S-nitrosoglutathione, S-nitrosocaptopril, glucose-S-nitroso-N-acetyl-DL-penicillamine-2, and the S-nitroso tripeptide acetyl-Phe-Gly-S-nitrosopenicillamine, exhibited different inhibitory activities toward the enzyme in a time- and concentration-dependent manner with second-order rate constants ($k_2/K_i$) ranging from 8.9 to 17.2 M⁻¹ s⁻¹. The inhibition of papain by S-nitrosothiol was rapidly reversed by dithiothreitol, but not by ascorbate, which could reverse the inhibition of papain by NO.$\text{NO}$. Incubation of the enzyme with a fluorescent S-nitroso probe (S-nitroso-5-dimethylaminonaphthalene-1-sulfonyle nitrile) resulted in the appearance of fluorescence of the protein, indicating the formation of a thiol adduct. Moreover, S-transnitrosylation in the incubation of S-nitroso inactivators with papain was excluded. These results suggest that inactivation of papain by S-nitrosothiols is due to a direct attack of the highly reactive thiolate (Cys²⁵) in the enzyme active site on the sulfur of S-nitrosothiols to form a mixed disulfide between the inactivator and papain.

NO is a newly discovered biological messenger that plays important roles in physiological and pathophysiological conditions such as septic shock, inflammation, and endothelium-dependent vasorelaxation (1, 2). Many effects of NO are based on its reaction with other important species. NO reacts with superoxide to make peroxynitrite (3). It also forms metal-nitrosyl complexes in the reaction of transition metals (4). The interactions of NO with sulfhydryl-containing molecules and enzymes have gained considerable importance (5, 6). In many biological systems, S-nitrosylation reactions, transferring NO from a NO donor to a protein sulfhydryl group, affect protein function. Targets for this type of modification, among others, are serum albumin (7), tissue-type plasminogen activator (8), transcriptional activators (9), gy-ceraldehyde-3-phosphate dehydrogenase (10), human immunodeficiency virus protease (11), and protein-phosphotyrosine phosphatase (12). Some cysteine proteases such as caspase-3 (13) and cathepsin K (14) have also been demonstrated to be inhibited by NO donors.

Cysteine proteases (EC 3.4.22.1–18) compose a large class of enzymes from plant, animal, and bacterial sources. They play important roles in various biological processes (for review, see Refs. 15 and 61). Since many disease states such as muscular dystrophy, inflammation, and rheumatoid arthritis are associated with elevated proteolytic activity of cysteine proteases, much attention has been paid to the rational design and synthesis of selective inhibitors of these types of enzymes (16–18). The active sites of cysteine proteases contain an essential cysteine sulfhydryl and histidine imidazole unit (19). The active thiol is highly sensitive to oxidation, which changes the enzyme’s properties and sensitivity to inhibitors and activators. It has been suggested that enzyme oxidation occurs in vivo and may represent an important mode of post-translational regulation of enzyme activity (20).

Papain, the most studied plant cysteine protease, shares many features with physiologically important mammalian cysteine proteases such as cathepsins B, H, L, and S and the calpains. The x-ray structure for human cathepsin B and earlier comparisons of the crystal structure of papain with models of cathepsins B and H demonstrated that the mammalian enzymes show nearly identical folding patterns to papain, especially around the active site (21). In the active site of papain, Cys²⁵ and His¹⁵⁹ are thought to be catalytically active as a thiolate-imidazolium ion pair. This enzyme should be susceptible to NO donors in a manner similar to other cysteine-containing enzymes. In fact, our previous reports (22, 23) have shown that papain can be efficiently inhibited by peptidyl or non-peptidyl N-nitrosoanilines (a novel class of stable NO donors). We demonstrated that the inactivation is due to the formation of a stable S-NO bond in the active site of papain (S-nitroso-Cys²⁵). Other authors, using (E)-ethyl-2-((E)-hydroxymino)-5-nitro-3-hexenamide and sodium nitroprusside as NO donors to inactivate papain, obtained the same results (24). S-Nitrosothiols are other important widely used NO donors. These compounds such as GSNO¹ may be the most relevant biological molecules to carry out nitrosation reactions under physiological conditions (25, 26). Some authors have even suggested that the actions of the endothelium-derived relaxing factor more closely resemble a low molecular weight nitrosothiol rather than the NO radical itself (27). In this study, we investigated the influence of S-nitrosothiols on papain. Our results show that S-nitrosothiols are efficient papain inhibitors and work through the formation of mixed disulfide species in the active-site cysteine (Cys²⁵) of the enzyme.

References:

1. The abbreviations used are: GSNO, S-nitrosoglutathione; SNAP, S-nitroso-N-acetyl-L- penicillamine; dansyl, 5-dimethylaminonaphthalene-1-sulfonyle DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic).
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EXPERIMENTAL PROCEDURES

Materials and General Methods

Enzyme, amino acids, amino acid derivatives, and all other chemicals, solvents, and reagents were obtained from commercial sources (Sigma, Aldrich, and Fluka). They were of the highest purity available and used without further purification unless otherwise noted. 1H and 13C NMR spectra were recorded on a Varian VRX 400S NMR apparatus. Silica Gel F254 plates (Merck) and Silica Gel 60 (70–230 mesh; Merck) were used in analytical TLC and column chromatography, respectively.

Synthesis of S-Nitroso Compounds 1–6 (See Fig. 1)

SNAP (1), GSNO (2), and S-nitrosoacetopipril (3) were prepared by the methods of Field et al. (29), Hart (29), and Loscalzo et al. (30), respectively. Glucose-SNAP-2 (4) was synthesized according to our previous method (31). Acetyl-Phe-Gly-S-nitrosothiocyanate (5) — Acetyl-Phe-Gly-OH (0.4 mmol) was reacted with 2-N-acetyl-3-S-triphenylmethyl-3-methylbutanolic acid methyl ester (1 eq) in 10 ml of dimethylformamide at 50 °C overnight with 1-(3-dimethylamino)propyl-3-ethylcarbodiimide hydrochloride (1.5 eq) and hydroxybenzotriazole (3 eq) as coupling reagents. The tripeptide was obtained in 30% yield. The free sulphydryl compound was afforded in 70% yield by protection of the triphenylmethyl group with trifluoroacetic acid (0.5 ml) in CH2Cl2. The target compound 5 was obtained in 85% yield by nitrosation of the free sulphydryl group with ethyl nitrite (4–5 eq) in MeOH/H2O at 0 °C. Compound 6: 1H NMR (CD3OD) δ 7.26–7.29 (m, 5H, 5.6 Hz), 7.05–7.08 (m, 5H, 5.6 Hz), 3.99–4.03 (m, 2H, 5.6 Hz), 3.76–3.79 (m, 2H, 5.6 Hz), 3.64–3.67 (m, 2H, 5.6 Hz), 3.33–3.36 (m, 2H, 5.6 Hz), 2.53–2.56 (m, 2H, 5.6 Hz), 2.30–2.33 (m, 2H, 5.6 Hz), 1.85–1.88 (m, 2H, 5.6 Hz), 1.25–1.28 (m, 2H, 5.6 Hz), 0.89–0.92 (m, 2H, 5.6 Hz).

Recovery of Enzyme Activity by Ascorbate and DTT

Active papain (0.5 mg/ml, 500 μl) was incubated with 500 μl of S-nitroso compounds (1 mM) or nitrosonium tetrafluoroborate (NOBF4; 6 mM) in 50 mM phosphate buffer, pH 6.2, 1 mM EDTA, and 0.1 mM NaCl at 25 °C for 60 min. The enzyme activity was totally abolished. The protein was then passed through a Sephadex G-25 column for purification. Fractions containing the protein were concentrated by centrifugal filtration (Centricon YM-3, Amicon, Inc.). The obtained inactive enzyme (0.5 mg/ml, 100 μl) was then incubated with 100 μl of l-ascorbic acid (20 mM) or DTT (10 mM), and the activity of papain was measured at different time intervals. Samples without inactivators served as controls.

Modification of Papain by Fluorescent Probe 6

Active papain (1 mg/ml, 600 μl) was incubated with compound 6 (2 mM, 600 μl) in 50 mM phosphate buffer, pH 6.2, 1 mM EDTA, and 0.1 mM NaCl at 25 °C until the papain was completely inactivated (<30 min). The protein was then passed through a Sephadex G-25 column to remove excess compound 6 and other low molecular weight reaction products. Fractions containing the protein (5 ml) were concentrated by centrifugation at 7000 rpm at 4 °C to produce a 1-m1 final solution. The fluorescence of the solution (0.5 mg/ml) was measured using a SPEX Fluor Max spectrometer. A control experiment including all the steps except for incubating papain with N-ethylmaleimide (0.1 mM) for 30 min before adding compound 6 was run and assayed in parallel.

Determination of S-Transnitrosation between Papain and S-Nitrosothiols

Papain activity was determined spectrophotometrically at 410 nm with a Hewlett-Packard 8453 UV/visible spectrophotometer using the chromogenic substrate 5-thio-2-nitrobenzoate anion (ε12ε = 13600 M−1 cm−1). The free thiol active papain (1 mg/ml) was titrated by 5-(octylidithio)-2-nitrobenzoate (1.2 mM) in 50 mM phosphate buffer, pH 6.2, 1 mM EDTA, and 0.1 mM NaCl at 25 °C. The thiol compounds were added to the enzyme and diluted into the enzyme assay solution containing the substrate. The residual enzyme activity was measured. A control preincubation solution containing all of the ingredients except for the inhibitor itself was run and assayed in parallel. These experiments were performed both in the absence and presence of Gly-Gly-Tyr-Arg.
The kinetic results presented in Table I indicated that S-nitroso compounds were moderate inactivators of papain. The second-order rate constants ($k_2/K_i$) for inactivation of papain by these compounds ranged from 17.2 to 8.9 $M^{-1} s^{-1}$. The values were substantially lower than those exhibited by other well-known papain inhibitors such as peptidyl diazomethanes. This result was partly attributable to the higher $K_i$ values of the S-nitrosothiols, whereas in the diazomethane case, the actual dissociation constant for the Michaelis-type complex was significantly lower due to the fast reversible chemical step preceding the alkylation step. The intrinsic reactivity of the nitrosothiol moiety toward the papain differed in these five compounds, of which the most potent was inhibitor 5, as we predicted. This result suggested that the Phe and Gly residues in inhibitor 5 contributed significantly to the stabilization of the inactivator-enzyme complex. However, by comparing the second-order rate constants for inhibition of papain by S-nitrosothiols with those exhibited by peptidyl N-nitrosoanilines (22), we can conclude that the functional S–NO residue is generally a more potent inactivator than the N–NO residue. By comparing the kinetic parameters of SNAP and glucose-SNAP-2, it appeared that the introduction of a sugar fragment decreased the inhibitory potency of SNAP. The same effect was observed for protein-tyrosine phosphatase (38).

**Identity of the Modification Site**—The next step was to identify the site implicated in the enzyme inactivation. Gly-Gly-Tyr-Arg is a competitive peptide inhibitor of papain. To determine whether the inhibitory effect of S-nitrosothiols on papain occurs due to modification at a similar or distinct site of action, we analyzed the inactivation of papain by S-nitrosothiols in the presence or absence of Gly-Gly-Tyr-Arg. The effects of Gly-Gly-Tyr-Arg alone and together with GSNO are shown in Fig. 4. A combination of GSNO and the tetrapeptide inhibited the enzyme by 42%, whereas individually, 75 and 96% inhibition was achieved, respectively. This result that the competitive inhibitor protected the enzyme from inactivation suggested that the action of S-nitrosothiols was directed to the active site of papain. Since papain has only one free sulfhydryl group (Cys25 in the active site of the enzyme), the data in Fig. 4 also suggested that inactivation of papain by S-nitroso compounds was due to the modification of Cys25 of the protein.

To further verify this conclusion, free thiol groups were titrated both before and after inactivation of the enzyme with S-nitroso compounds (data not shown). We found that only <0.05 free thiol groups/enzyme molecule were titrated after incubating the enzyme (1 mg/ml) with S-nitrosothiols (0.5 mM) for 30 min, whereas 0.81 free thiol groups were titrated in the uninhibited enzyme. These results also indicated that the sulfhydryl group in the active site of papain (Cys25) was involved in the enzyme inactivation and that the modification of this sulfhydryl group by inactivators was complete.

**Mechanism of Inhibition**—It is well known that the activities of many cysteine-containing enzymes are modulated by an NO-induced mechanism (39). The present dogma for the mechanism of inactivation of critical thiol-containing enzymes by NO or NO donors is the formation of an S-nitroso adduct (40, 41). This species, which has been identified by spectroscopic and colorimetric quantitation techniques in a number of proteins, is reducible to the free thiol by DTT with the recovery of enzyme activity (42). This latter criterion has been used to postulate the presence of an S-nitrosothiol group in recent studies on the effects of NO donors on enzymes, sometimes in the absence of direct evidence (43–45). If NO donors used in the inactivation of cysteine-containing enzymes were compounds that release only nitric oxide as the inactivating reagent in the incubation (such as diazeniumdiolates, sodium nitroprusside,
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**FIG. 3.** Kitz-Wilson plot for the inhibition of papain by GSNO.

**TABLE I**

Kinetic parameters for the inactivation of papain by S-nitrosothiols

| S-Nitroso compounds | $k_i$ | $K_i$ | $k_i/K_i$ |
|---------------------|-------|-------|-----------|
| 1                   | 0.318 | 0.356 | 14.9      |
| 2                   | 0.202 | 0.305 | 11.1      |
| 3                   | 0.239 | 0.449 | 8.9       |
| 4                   | 0.477 | 0.618 | 12.9      |
| 5                   | 0.091 | 0.096 | 17.2      |

**FIG. 4.** Inhibition of papain by GSNO and Gly-Gly-Tyr-Arg.

Papain (0.2 mg/ml, 100 μl) was treated with GSNO (0.25 mM, 100 μl) or Gly-Gly-Tyr-Arg (0.1 mM, 100 μl) or both (100 μl) for 30 min at 25 °C in 50 mM phosphate buffer, pH 6.2, 1 mM EDTA, and 0.1 mM NaCl. At various time intervals, aliquots were removed and assayed for residual activity.

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3-morpholinosydnonime, (E)-methyl-2-((E)-hydroxyiminio)-5-nitro-6-methoxy-3-hexenamide, and N-nitrosoaniline derivatives, the modification of enzyme may be caused by the S-nitroso protein in many cases. Sometimes, the formation of protein sulfenic acid may be possible due to the oxidation of thiol groups in the protein by these inactivators (14, 46, 47). However, if an S-nitrosothiol with another thiol is complex (48, 49), it would not only produce the transnitrosated product, but also give rise to mixed disulfides in many cases. Both modifications would lead to the inactivation of cysteine-containing enzymes that could be reversed with DTT (Scheme 1). Thus, the reactivation of the enzyme by DTT should not be taken alone as evidence for the formation of an S–NO bond.

Recently, it was reported that ascorbate can promote NO release from S-nitrosoalbumin and GSNO in blood plasma (50). In another study using plasma and fractions of liver and kidney extracts, it was also claimed that ascorbate promoted the decomposition of GSNO (51). Another report in the biological literature found glutathione and nitrite as the products of the reaction of GSNO with ascorbate (52). Ascorbate and the thiol group of serum albumin are the plasma components mainly involved in the release of NO. However, in contrast to the thiol-dependent release, which is known to induce the formation of disulfides, the ascorbate-dependent release of NO from S-nitroso compounds (such as GSNO) resulted in the formation of free sulfhydryl groups. Williams’ group (53, 54) examined in detail the effect of ascorbate on S-nitroso compound decomposition. Their results showed that there were generally two separate reactions of ascorbate with S-nitrosothiols, both of which led to NO formation: (i) when ascorbate acted as a reducing agent for Cu²⁺ (a well-known reaction), which was dominant at low ascorbate concentrations (typically up to $1 \times 10^{-5}$ M); or disulfide formation; and was totally halted by the addition of EDTA; and (ii) when ascorbate acted as a nucleophile and underwent electrophilic nitrosation by S-nitroso compounds, leading to NO and thioli formation. This reaction was not affected by the presence of Cu²⁺ or EDTA and was dominant at higher ascorbate concentrations (typically $1 \times 10^{-3}$ to $1 \times 10^{-2}$ M). The above results suggested that under our experimental conditions (with EDTA), ascorbate could be used as an efficient reagent to distinguish the formation of S–NO and –S–S bonds in the protein. If the modification of the free thiol group in papain was to form S–NO protein, the addition of ascorbate could reduce the S-nitrosothiol to free thiol, and hence, the activity of enzyme would be recovered. If the modification of the free thiol group in papain was to form a mixed disulfide S–S–S–, the addition of ascorbate could not reduce it to free thiol. Then, the activity of enzyme would not be recovered (Scheme 2).

To identify whether the S-nitrosothiol-induced modification of the enzyme was due to the formation of an S–NO or S–S bond, the modified enzyme was separated from unbound S-nitrosothiol by rapid gel filtration using a Sephadex G-25 column equilibrated with reaction buffer. The purified enzyme was then incubated with ascorbate or DTT at 25 °C. The recovered enzyme activity was measured. Typical results using GSNO as the inactivator are shown in Fig. 5. The activity of papain dropped to ~4% after incubating the enzyme with GSNO for 60 min. The addition of ascorbate had no distinct effect on the recovery of enzyme activity, but when the inactive enzyme was incubated with DTT, its activity increased rapidly. For a period of time, the activity was even higher than the original activity of the enzyme. (Active papain may be partly oxidized during purification or storage. Thus, the enzyme used in this experiment did not reach its maximum activity; but with excess DTT, the enzyme could obtain the maximum activity.) The different effects of DTT and ascorbate on the inactivated enzyme suggested that the inactivation of this cysteine protease with S-nitrosothiol led to the formation of a disulfide bond.

To further ensure our conclusion, we also used this procedure to investigate the effect of NOBF₄ on the enzyme. NOBF₄ is an exclusively NO⁻-releasing NO donor that forces S-nitroso-protein generation via a nitrosation reaction. Thus, incubation of papain with NOBF₄ would lead to the formation of an S–NO bond in the active site of papain. The effects of NOBF₄ on papain are shown in Fig. 6. Incubation of papain with NOBF₄ inactivated the enzyme activity completely (>92%) in 30 min and then remained unchanged. To identify whether the NOBF₄-induced modification of papain was due to the formation of S-nitroso protein, the inactive enzyme was purified by rapid gel filtration again. This inactive protein showed a UV-visible spectrum absorption maximum in the 330–370-nm range.
wavelength range, characteristic of S-nitrosothiol. It had the same spectroscopic characteristics as those we observed upon incubation of this enzyme with N-nitroso inhibitors (22). Ascorbate and DTT were then separately used to recover the enzyme activity. As we predicted, the addition of DTT recovered the enzyme activity at an even higher level than its original activity (the same reason as mentioned for Fig. 5). In contrast to the effect of GSNO, upon incubation of the inactivated enzyme with ascorbate for 40 min at 25 °C, ~82% of the enzyme activity was recovered. This result indicated that ascorbate was an efficient reducing agent for S-nitroso protein. It enhanced the regeneration of the free sulfhydryl group (Cys25) in the enzyme.

Previous reports have demonstrated that S-nitrosothiols can produce NO\(^{--}\), which in turn leads to transnitrosylation, as does NOBF\(_4\). Kinetics of such S-transnitrosylation from one thiol to another thiol have been published (34, 55–57). However, our results showed clearly different effects of GSNO and NOBF\(_4\). Kinetics of such S-transnitrosylation processes were good probes for studying the interaction of S-nitrosothiols with the protein’s free sulfhydryl group. Such a method provided direct and observable evidence for the formation of a mixed disulfide bond between the protein and low molecular weight S-nitrosothiol.

**Exclusion of S-Transnitrosation in the Enzyme Inactivation**—Recently, from their study on the inactivation of glyceraldehyde-3-phosphate dehydrogenase by GSNO, Mohr et al. (59) suggested that if the cysteine of the enzyme was activated by a nearby histidine to form a strong nucleophile in its active site, this strong nucleophile (histidine-activated thiolate) could directly attack the S–NO bond of S-nitrosothiol, leading to the formation of a mixed disulfide bond. If the enzyme’s thiol group was not activated by a histidine group, as in alcohol dehydrogenase, the sulfhydryl group would have a low nucleophilicity and would not be able to break the S–NO bond of S-nitrosothiol. These kinds of enzymes were more likely to undergo an S-transnitrosation process with S-nitrosothiol inhibitors. However, Williams and co-workers (56, 57) have demonstrated that S-nitrosothiols to undergo S-transnitrosation with the protein’s free sulfhydryl group. This method provided direct and observable evidence for the formation of a mixed disulfide bond between the protein and low molecular weight S-nitrosothiol.

**Formation of Mixed Disulfides from Fluorescence Evidence**—To obtain direct evidence of the formation of an S–S-bond in this protein, compound 6 was used as an inactivator in our study. This new type of S-nitrosothiol, which coupled the functional S–NO group with the fluorescent molecule, was recently prepared for detecting intracellular thiols and S-nitrosothiols (58). Thus, it could be used as a fluorescent probe to identify the modified form of Cys25 in papain. After incubating active papain with compound 6 for 30 min at 25 °C, the enzyme was inactivated completely. The protein was then purified on a Sephadex G-25 column as described under “Experimental Procedures.” The solution of this inactivated protein gave a strong fluorescence signal (\(\lambda_{em} = 345\) nm and \(\lambda_{ex} = 510\) nm) (Fig. 7, trace B). However, if the free sulfhydryl group of the enzyme was blocked by N-ethylmaleimide before incubation with compound 6, the protein solution obtained from the same procedure did not exhibit any fluorescence (Fig. 7, trace A). Even with a hand-held UV lamp, one could observe the difference in the treated protein solutions from the two procedures. The solution of the modified protein (Fig. 7, trace B) showed green fluorescence, whereas the solution shown in trace A did not show any fluorescence. These results indicated that fluorescent S-nitrosothiol compounds were good probes for studying the interaction of S-nitrosothiols with the protein’s free sulfhydryl group. This method provided direct and observable evidence for the formation of a mixed disulfide bond between the protein and low molecular weight S-nitrosothiol.
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**FIG. 7.** Fluorescence spectra of inactivated papain. A, active papain (1 mg/ml) was pretreated with N-ethylmaleimide (0.1 mM) for 30 min at 25 °C to block the free thiol of the protein. Then, it was incubated with compound 6 (2 mM) for another 30 min. The protein was purified and concentrated as described under “Experimental Procedures.” The fluorescence of the protein (0.5 mg/ml) was measured. B, active papain (1 mg/ml) was incubated with compound 6 (2 mM) for 30 min. It was then purified and concentrated. The fluorescence of the protein (0.5 mg/ml) was measured.

Based on these facts, we suspected that when the histidine-activated thiolate group (Cys25) in papain reacts with S-nitroso inhibitors, the S-nitrosylation enzyme is the initial intermediate produced, but the mixed disulfide protein is the final product detected.

To find out whether S-transnitrosation occurred in the incubation of papain and S-nitroso inhibitors, a sensitive procedure to monitor the S-transnitrosation of protein cysteine with low molecular weight S-nitrosothiols was used in this study. The procedure (34) is based on the low reactivity of DTNB with molecular weight S-nitrosothiols. The reaction (Scheme 3) is based on the low reactivity of DTNB with S-nitrosothiols, which would react rapidly with DTNB to give 5-thio-2-nitrobenzoate anion, which could be conveniently monitored. In our experiment, the concentration of the free thiol in papain was 8.5 μM. When the protein underwent a transnitrosation with these simple S-nitroso compounds, it would produce ~8.5 μM simple thiol compounds. Upon incubation of 8.5 μM simple thiols such as GSH with DTNB, the UV absorption at 412 nm reached 0.112 ± 0.008 as soon as the compounds were mixed. However, the A412 changes obtained from the incubation of papain with compounds 1–5 ranged only from 0.006 (2) to 0.013 (5) (Table II). This indicated that <10% of the S-transnitrosation product existed in the inactivation reaction. These data clearly showed that S-transnitrosation was not the favorite process in the inactivation of this cysteine protease by the simple S-nitroso inhibitors.

**Conclusion—S-Nitrosothiols such as GSNO display several biological effects, including platelet deactivation, immunosuppression, relaxation of vascular smooth muscle cells, and neurotransmission (6).** Micromolar concentrations of nitrosothiols have been detected in plasma and bronchial lavage fluid (60). On the basis of these observations, S-nitrosothiols may be the bioreactive intermediates that mediate some of the effects of NO. Nonetheless, the mechanisms by which nitrosothiols modulate cell function and alter protein structure are not well understood. Many previous papers suggested that the biological effects of nitrosothiols were due to their tendency to liberate free NO or to modify other functional cysteine-containing proteins through S-transnitrosylation. However, several recent reports have demonstrated that inactivation of some cysteine-containing enzymes is due to the formation of mixed disulfides (14, 59). Mass spectroscopy techniques and radioactive mark-ers were used to obtain the evidence. In this study, two facile methods (ascorbic acid and a fluorescent S-nitroso probe) were used to identify the formation of mixed disulfide protein. Because we excluded the possibility of S-transnitrosation in the process, we conclude that S-nitrosothiol is directly attacked by the thiolate group (Cys25) in the activation of the enzyme. This conclusion is consistent with previous reports (14, 59). These results suggest that, in addition to their well studied roles as nitrosating agents, S-nitrosothiols can also modify protein structure and function by inducing mixed disulfides and intramolecular disulfides, which may represent a novel means of NO-mediated redox signaling.

**TABLE II**

| S-Nitroso compounds | A412 changes |
|---------------------|-------------|
| 1                   | 0.010       |
| 2                   | 0.006       |
| 3                   | 0.008       |
| 4                   | 0.011       |
| 5                   | 0.013       |

**SCHEM 3.** RSNO, S-nitrosothiol; SNO, S-nitroso compound; TNB, 5-thio-2-nitrobenzoate anion.
