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Monitoring Reactions of Nitric Oxide with Peptides and Proteins by Electrospray Ionization-Mass Spectrometry*

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Recent studies have demonstrated the biological importance of the interaction of nitric oxide (NO) with proteins. Protein-associated targets of NO include heme, Cys, and Tyr. Electrospray ionization-mass spectrometry was used to monitor the results of exposure of model peptides and an enzyme to NO under different conditions and thus addressed aspects of NO-protein interactions. The molecular mass of a decapeptide containing a single Cys residue increased by 29 Da upon treatment with NO under aerobic and acidic conditions, consistent with the substitution of one NO moiety. The mass of reduced somatostatin, a peptide containing two Cys residues, increased by 58 Da, consistent with the substitution of two NO moieties. These substitutions were prevented by pretreatment of the peptides with *N*-ethylmaleimide. The strength of the nitrosothiol bond was examined by varying the amount of energy applied to the peptide ions and indicated a labile species. Cys residues were very rapidly nitrosated, while other reactions were observed to occur at much slower rates. These include the further oxidation of nitrosothiol to sulfonic acid and nitration of Tyr. Peptides treated with NO at physiological pH were observed to undergo dimerization as well as nitrosation. These studies were extended to the enzyme $p21^{ras}$, whose activity has been postulated to be modulated by nitrosothiol formation, and revealed the formation of a single nitrosothiol on $p21^{ras}$ upon NO treatment. These data suggest that electrospray ionization-mass spectrometry allows for quantitation and characterization of nitrosothiol bonds in peptides and proteins.

The interaction of NO[1] with proteins is known to play a critical role in regulating blood pressure, host defense, and neurotransmission (1, 2). Known targets of NO on proteins are Cys and Tyr residues (3, 4) and metals such as heme-Fe^{2+} (5, 6). Although much work has been performed characterizing the NO-Fe^{2+} interaction (6), there is considerably less data on the result of NO-Cys interactions. One reason is the labile nature of nitrosothiol (RSNO) bonds. Nitrosothiol bonds are thought to occur via substitution by NO^+, or other nitrogen oxides, on free sulfhydryl groups (3).

Nitrosothiol formation is considered a likely outcome of NO-Cys interactions at acidic pH (3, 7). In fact, the function of several proteins has been postulated to be modulated by NO through formation of RSNO, including $p21^{ras}$ (8). At physiological pH, RSNO formation is thought to be less favorable, although RSNO on proteins has been found *in vivo* (9). Furthermore, it has been suggested that transnitrosation reactions on vicinal thiols can occur, leading to disulfide formation (3).

Electrospray ionization-mass spectrometry (ESI-MS) is a very accurate method of determining molecular mass (10), and conditions can be varied such that the protein of interest is subjected to gentle perturbations during analysis in an effort to preserve labile structures. We employed ESI-MS to monitor the results of exposure of model peptides and an enzyme to NO under different conditions and thus addressed aspects of NO-protein interactions.

MATERIALS AND METHODS

Source of Peptides and $p21^{ras}$—A peptide (KNNLKECGLY, mass = 1181.4 Da) corresponding to the C terminus of the G protein $G_{i\alpha 1}$ was commercially synthesized. Somatostatin was from Sigma (catalog no. S-9129). Purified bacterially expressed $p21^{H\text{-}ras}$ was kindly provided by Dr. Daniel Manor, Department of Pharmacology, Cornell University (Ithaca, NY).

Preparation of Nitric Oxide Solutions—NO solutions were prepared as we previously described (11). Briefly, a solution of 20 mM ammonium bicarbonate solution, pH 8.0, in a rubber-stoppered tube was sparged for 15 min with N_2 and then 15 min with NO gas (Matheson Gas, East Rutherford, NJ). This resulted in a saturated solution of NO (1.25 mM). This solution also contained higher oxides of NO that were not quantified.

Electrospray Ionization-Mass Spectrometry—The electrospray ionization mass spectra were obtained on a Finnigan-MAT TSQ-700 triple quadrupole instrument. Unless otherwise indicated, peptides and protein samples were electrosprayed from acidified (acetic acid) 50% methanolic solutions (pH 3.0), and the concentrations of the analyte electrospray solutions were in the range of 10–20 μM. The measurements of pH were made with a PHM 95 pH meter (Radiometer, Copenhagen) calibrated in aqueous solutions. No corrections were applied for the pH measurements of solutions containing methanol. The analyte solutions were infused into the mass spectrometer source using a Harvard syringe pump (model 24000–001) at a rate of 3 μl/min through a 100-μm (inner diameter) fused silica capillary. The positive ion spectra obtained were an average of 16 scans and were acquired at a rate of 3 s/scan. The ion signals were recorded by a Finnigan ICIS data system operated on a DECstation 5000/120 system. The reconstructed molecular mass profiles were obtained by using a deconvolution algorithm (FinniganMAT).

In ESI-MS experiments, the degree of energy input into the peptide and protein ions can be controlled by two independent variables (12). One variable is the temperature of the capillary transfer tube. The other variable is the potential difference between the capillary transfer tube and the tube lens of the mass spectrometer (ΔV). The majority of mass spectra were obtained at a mass resolution that did not resolve the individual isotopic components. However, when more detailed information was required, the mass resolution was increased sufficiently to resolve the individual isotopic components.

RESULTS

Determination of Peptide Nitrosothiol Content Using ESI-MS—The regulation of protein and enzyme function by NO through nitrosation of critical Cys residues is emerging as an

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[1] The abbreviations used are: NO, nitric oxide; RSNO, nitrosothiol; ESI-MS, electrospray ionization-mass spectrometry; NEM, *N*-ethylmaleimide; DTE, dithioerythritol.

important control switch in NO signaling (3, 7, 8, 13). The RSNO bond is of a labile nature and thus difficult to study and quantify. Therefore, we applied ESI-MS to characterize NO-peptide interactions under conditions where RSNO formation is favored. A peptide (KNNLKECGLY, mass = 1181.4 Da) corresponding to the C terminus of the G protein $G_{i\alpha 1}$ was either untreated (Fig. 1*A*) or treated with (Fig. 1*B*) 10-fold molar excess NO. Treatment with NO for 5 min at pH 3.0 resulted in detection of a new species with a mass 29.0 ± 0.2 Da greater than the starting peptide. The observed difference of 29 Da corresponds to the molecular mass of NO (30 Da) minus the mass of the substituted proton (1 Da). This mass difference of 29 Da shows unequivocally that treatment of this peptide with NO at pH 3.0 resulted in a substitution reaction. Pretreatment of this peptide with *N*-ethylmaleimide (NEM), an irreversible thiol-binding reagent (14), led to a new species with a mass equal to that of peptide + NEM (Fig. 1*C*). Subsequent treatment of this complex with NO did not yield a species with higher mass (Fig. 1*D*), indicating that the NEM modification blocked reaction of NO with the peptide. This demonstrates that NO substitution had originally occurred on the Cys residue (Fig. 1*B*). Treatment of peptide with NO at pH 7.8 also led to nitrosothiol formation but to a much lesser extent. The major product at pH 7.8 was peptide dimer (Fig. 1*E*).

Determination of Reaction Kinetics by ESI-MS—Modification of proteins by NO to yield tyrosine nitration (Tyr-NO_2) or sulfonic acid (SO_3H) has been suggested (15, 16). We examined the peptide-NO adducts formed after various times of exposure of the test peptide (KNNLKECGLY) to NO using ESI-MS. After 5 min of exposure to NO, nitrosothiol was the major product formed at pH 3.0 (Fig. 1*B*), which persisted to 45 min (data not shown). However, after 2 h and more prominently at 7 h, two new species arose. One had an increased mass of 49 Da greater than that of the starting peptide, likely corresponding to a sulfonic acid derivative. The other had an increased mass of 94 Da greater than that of the starting peptide, likely corresponding to a peptide with both Tyr-NO_2 (45 Da) and SO_3H (Fig. 2, *A* and *B*). After 24 h, very little starting peptide and nitrosothiol remained (Fig. 2, *C* and *D*), and the majority of the peptide had either SO_3H or both Tyr-NO_2 and SO_3H (Fig. 2, *C* and *D*). Formation of SO_3H was confirmed by pretreatment of the peptide with NEM. Treatment of the peptide-NEM complex (mass = 1307 Da) for 70 h with NO yielded both the starting complex and a peptide complex with a mass increased by 45 Da (mass = 1352 Da, Fig. 2*E*), indicating that only nitration occurred. Neither sulfonic acid nor nitration and sulfonic acid derivatization was seen (expected mass = 1356 and 1401 Da, respectively). A number of low abundance reaction products was also observed. Thus, ESI-MS detected nitrosation, nitration, and sulfonic acid formation on a peptide upon exposure to NO. The order of reactivity was: RSNO ≫ Tyr-NO_2 = SO_3H.

Using ESI-MS to Study the Chemistry of Nitrosothiols—We studied the nature of the peptide-RSNO bond by altering the temperature of the metal capillary transfer tube. Our indicated temperatures of the capillary transfer tube do not reflect the temperatures of the peptide and protein ions but rather provide a relative measure of energy input. We found that at 125 °C (Fig. 1*B*) or 150 °C (Fig. 3*A*), the nitrosated peptide was readily detected. At 175 °C, approximately 50% of the nitrosothiol bonds was broken (Fig. 3*B*), and at 200 °C nitrosothiols were undetectable (Fig. 3*C*). The observed mass difference between the nitrosated peptide and the heat-induced decomposition product was 30.1 ± 0.2 Da. This mass difference of 30 Da demonstrates that homolytic decomposition of the RSNO bond occurred, yielding the thiyl radical (*i.e.* R–S$^{\cdot}$). These results indicate that ESI-MS can also be used to determine the relative strength of RSNO bonds

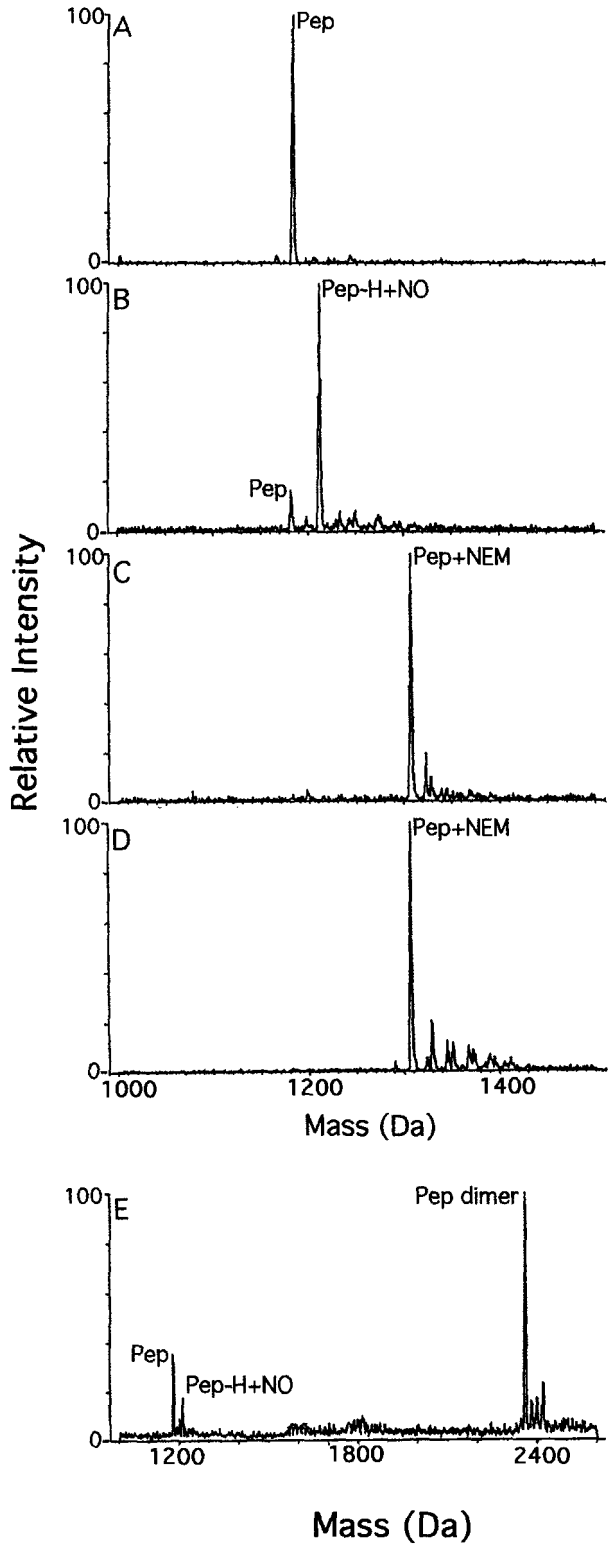

FIG. 1. **Determination of peptide nitrosothiols using ESI-MS.** *A*, peptide (*Pep*, 100 μM, in 5 μl of 20 mM ammonium bicarbonate, pH 7.8) was mixed with 20 μl of H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0); *B*, NO (100 μM) was added to peptide and immediately mixed with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0); *C*, NEM (1 mM) was added to peptide for 5 min at 22 °C and then mixed with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0); *D*, NO (100 μM) was added to the same preparation as in *C* and immediately mixed with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0); *E*, same preparation as in *B* but mixed with H_2O:CH_3OH (1:1, pH 7.8). Samples were left at 22 °C for 5 min prior to mass spectrometric analysis. T = 125 °C; ΔV = 80. Satellite peaks arise from adventitious cation adduction.

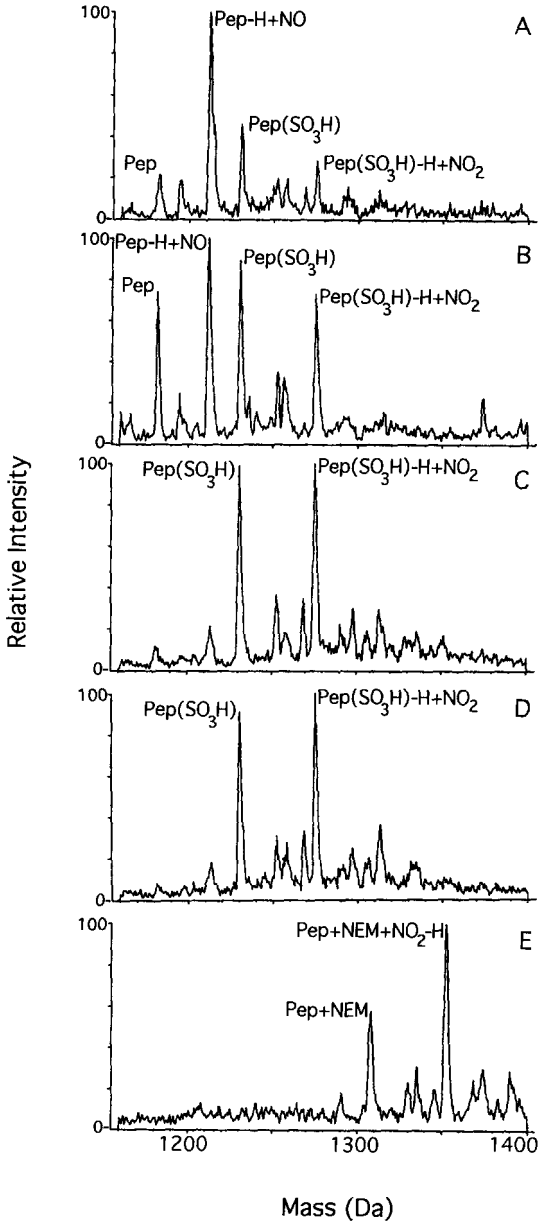

FIG. 2. **Kinetics of reaction of peptide with NO.** *A*, NO (100 μM) was added to peptide (*Pep*) for 2 h and then mixed with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0); *B*, same treatment as in *A* but for 7 h; *C*, 24 h; *D*, 70 h; *E*, NEM (1 mM) was added to peptide for 5 min at 22 °C, and then NO (100 μM) was added and the sample left at 22 °C for 70 h prior to mixing with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0). T = 125 °C; ΔV = 80.

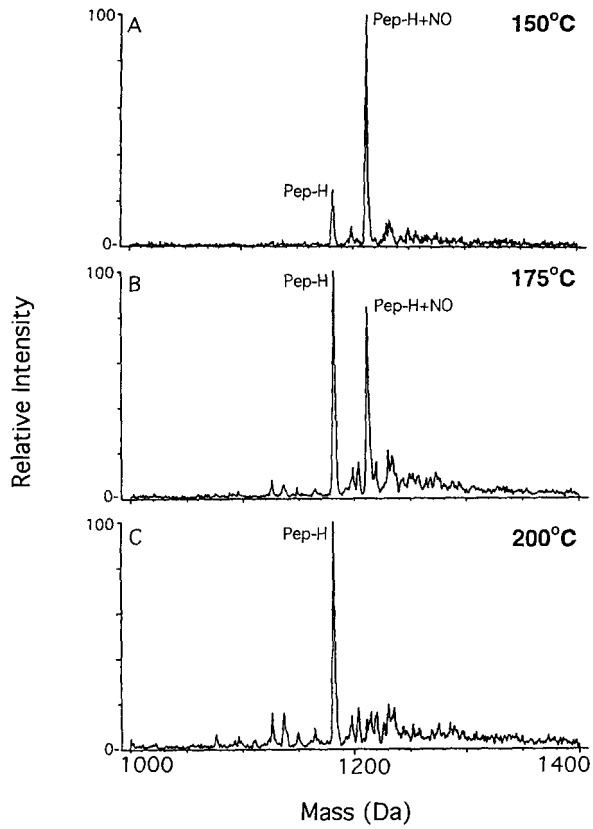

FIG. 3. **Effect of capillary transfer tube temperature on nitrosothiols.** NO (100 μM) was added to peptide (*Pep*) for 5 min and immediately mixed with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0) and infused at the indicated capillary temperatures. ΔV = 80.

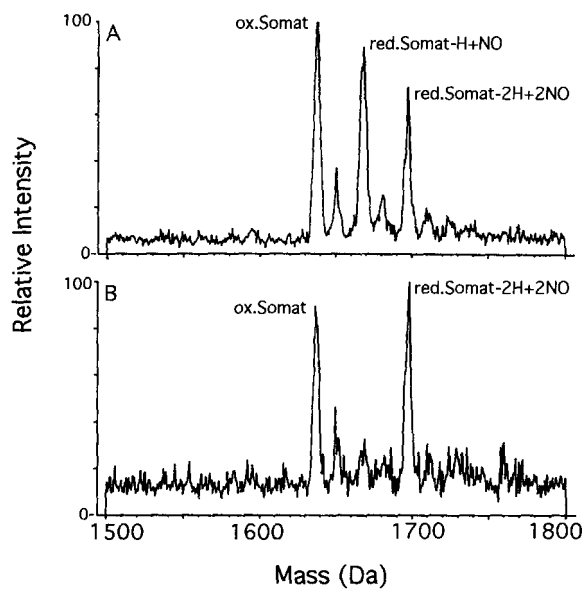

FIG. 4. **Effect of varying NO concentration on nitrosothiol formation.** Somatostatin was first treated with DTE overnight and then with NO for 5 min (*A*, 1:5:10; *B*, 1:5:30, somatostatin:DTE:NO, mol/mol/mol) prior to mixing with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0). T = 125 °C; ΔV = 80. *Somat*, somatostatin; *ox.*, oxidized; *red.*, reduced.

and that gentle conditions are required for their detection.

Concentration-dependent Nitrosation of Reduced Somatostatin—Somatostatin is a 1637.7-Da peptide hormone containing two Cys residues that are normally disulfide-linked. We reduced somatostatin with dithioerythritol (DTE) overnight at room temperature (somatostatin:DTE, 1:5). We then added NO in either a 1:5:10 (somatostatin:DTE:NO) ratio (Fig. 4*A*) or a 1:5:30 ratio (Fig. 4*B*). As seen in Fig. 4*A*, addition of a slight excess of NO yielded oxidized somatostatin and species of increased mass consistent with one or two RSNO bonds. When NO is added in greater excess, analysis by ESI-MS detects oxidized somatostatin and somatostatin with two RSNO bonds (Fig. 4*B*). Pretreatment of reduced somatostatin with NEM yielded a species of 250-Da increased mass (arising from modification of reduced somatostatin by 2 NEM molecules) and prevented NO from altering the mass (data not shown), indicating that RSNO bonds are responsible for the increased molecular mass observed in Fig. 4, *A* and *B*. NO had no effect on native somatostatin after treatment for 10 min (data not shown). These data demonstrate that nitrosothiols can form in a concentration-dependent manner and that the stoichiometry

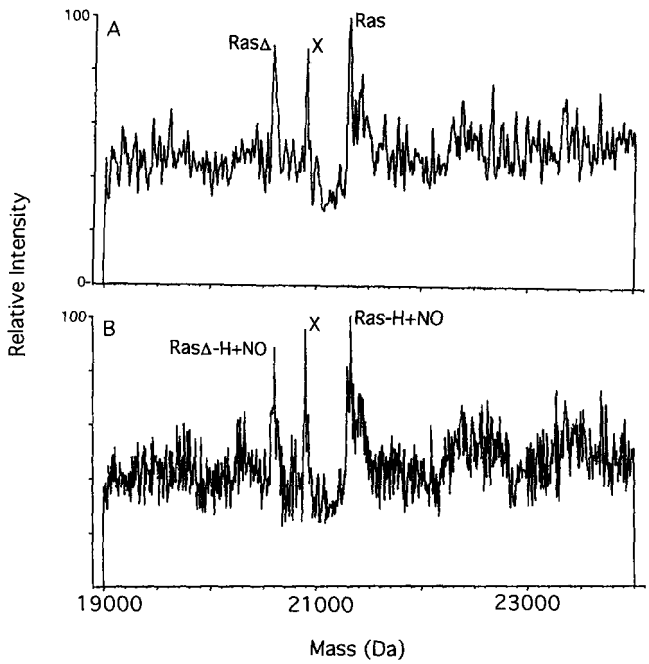

FIG. 5. **ESI-MS of NO-treated p21^{ras}.** p21^{ras} (20 μM) was treated without (A) or with (B) NO (100 μM) for 5 min prior to mixing with H_2O:CH_3OH:CH_3COOH (1:1:0.05, v/v/v, pH 3.0). T = 125 °C; ΔV = 80. *Ras*Δ indicates truncated p21^{ras}; *Ras* indicates full-length p21^{ras}; and *X* indicates an unidentified impurity, whose mass does not shift upon NO treatment.

of modification is easily determined with ESI-MS.

Nitrosothiol Formation on an Enzyme—We have previously identified G proteins, and particularly p21^{ras}, as potential targets of NO (8, 11, 17). Therefore, we utilized ESI-MS to determine if NO could modify p21^{ras} through formation of nitrosothiol bonds. Purified bacterially expressed p21^{ras} yielded three major species on ESI-MS (Fig. 5*A*). The species with a molecular mass of 21,296 ± 2 Da corresponds to that of the native enzyme and the species 20,575 ± 2 Da to that of the enzyme from which 7 C-terminal amino acids have been lost during purification. Treatment of our p21^{ras} preparation for 5 min with 4-fold excess NO resulted in an increase of both species by 30 ± 3 Da (Fig. 5*B*). These modified species are not stable at a capillary temperature of 200 °C, indicating their labile character. Bacterially expressed p21^{ras} has five reduced Cys residues available for nitrosation. The present ESI-MS studies indicate that only one Cys is nitrosated. Thus, this technique also allows for quantitation of RSNO on proteins.

DISCUSSION

We have used ESI-MS to identify, quantify, and characterize modifications of peptides and protein as a result of exposure to NO. In a peptide containing one Cys residue, a nitrosothiol bond was identified and its thermodynamic properties examined by altering the temperature of the electrospray capillary transfer tube. The bond was found to be labile, and therefore its detection requires the relatively gentle conditions used in these studies. It should also be possible to calculate the ΔG of this bond relative to other protein modifications known to regulate protein function (*e.g.* phosphorylation). At physiological pH, nitrosothiol formation occurred to a lesser extent than at acidic pH, and peptide dimer became the predominant product. Thus, NO can facilitate transnitrosation reactions as has previously been suggested (3, 18).

The high accuracy of molecular mass determination using ESI-MS (error ≈ 0.02% or ±0.2 Da in our peptide of 1181.4 Da) permitted us to determine that the NO-modified peptide differed from that of the starting peptide by 29.0 ± 0.2 Da. Therefore, we have clearly identified that an RSNO formed concurrent with removal of the RSH proton.

Additional chemical modifications were also identified, which include nitration and sulfonic acid formation. These latter modifications formed at a much slower rate than nitrosothiols, which were detected in less than 5 min. Formation of SO_3H and Tyr-NO_2 was first seen after 2 h of treatment and was fully developed after 7 h. The order of reaction in our model peptide was RSNO ≫ Tyr-NO_2 = SO_3H. Therefore, nitrosothiol formation would likely be a preferred regulator of protein function as compared with other modifications due to NO.

The number of nitrosothiols formed on reduced somatostatin, a peptide hormone with two Cys residues, was found to be dose-dependent and controlled by the amount of reducing agent present. This suggests that in the cell, where endogenous modulators of redox state such as glutathione exist, formation of RSNO on proteins may be regulated. Furthermore, p21^{ras}, a 21-kDa enzyme whose regulation by NO has been postulated to be controlled by nitrosation (8), was demonstrated to have one nitrosothiol formed upon treatment with NO. This enzyme has five free Cys residues, and, therefore, the formation of only one RSNO suggests the possibility that nitrosation of this enzyme occurs at a specific site.

The regulation of some heme-containing enzymes, such as guanylyl cyclase, by NO is known to occur through binding of NO to the Fe^{2+} of heme (6). Recently, evidence has emerged that suggests that NO may also regulate certain heme-containing enzymes, such as cyclooxygenase-1 (prostaglandin H synthase-1), through nitrosothiols (19). We are currently exploring the interaction of NO with heme-containing enzymes using ESI-MS.

Current methods of colorimetric detection of nitrosothiols require large amounts of sample (20) and require acidification of the sample. Other spectroscopic methods exist including UV, IR, and NMR techniques. Again sample amount, purity, and quantitation are serious difficulties associated with these methods. ESI-MS can detect nitrosothiols in very small amounts of sample and can determine the stoichiometry of substitution. Furthermore, this method allows coupling to high pressure liquid chromatography, which should enable on-line mass spectrometric peptide mapping of sites of RSNO formation.

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