Reversible S-Nitrosation and Inhibition of HIV-1 Protease†

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ABSTRACT: Nitric oxide (•NO) is a short-lived free radical with many functions including vasoregulation, synaptic plasticity, and immune modulation and has recently been associated with AIDS pathology. Various pathophysiological conditions, such as viral infection, trigger inducible nitric oxide synthase (iNOS) to synthesize NO in the cell. NO-derived species can react with thiols of proteins and form nitrosothiol adducts. HIV-1 protease (HIV-PR) contains two cysteine residues, Cys67 and Cys95, which are believed to serve a regulatory function. We have found that HIV-PR is inactivated by nitric oxide produced in vitro by NO donors and by iNOS. Sodium nitroprusside inhibited HIV-PR by 70%, and *S*-nitroso-*N*-acetylpenicillamine completely inhibited the enzyme. Furthermore, iNOS generated sufficient NO to inhibit HIV-PR activity by almost 90%. This inactivation was reversed by the addition of reducing agents. Treatment of HIV-PR with NO donors and ritonavir (a competitive peptide inhibitor) indicates that NO exerts its effect through a site independent of the active site of HIV-PR. Using electrospray ionization mass spectrometry, we found that NO forms *S*-nitrosothiols on Cys67 and Cys95 of HIV-PR which directly correlate with a loss of activity. These data indicate that NO may suppress HIV-1 replication by directly inhibiting HIV-PR.

The HIV-1 protease (HIV-PR)[1] plays a critical role in the life cycle of the HIV-1 virus. It cleaves gag protein and gag−pol fusion protein to individual structural proteins and enzymes, which are essential for the replication and maturation of the virus. The HIV-PR consists of 99 amino acids, has Asp-Thr-Gly in the catalytic site, and is a member of the aspartate protease family of proteins (*1*). It is active as a dimer with the amino acid triad of each monomer contributing equally to the catalytic process. HIV-PR autoprocesses itself from the polyprotein (*2*) and releases individual components during or immediately after budding of the virus from infected cells (*3*, *4*).

Cysteine modification affects the activities of different cytosolic and membrane proteins of bacterial, viral, and eukaryotic origin (*5*, *6*). Two cysteine residues, Cys67 and Cys95, are present at the surface of HIV-PR and are relatively conserved among different viral isolates found in HIV-1-infected patients (*7*, *8*). It has been suggested that these residues serve a regulatory role in the catalytic function of HIV-PR (*9*−*11*). Extensive studies have revealed that chemical modification of Cys95, present at the dimer interface, has a profound inhibitory effect on the activity of HIV-PR (*12*, *13*). By contrast, mutations of cysteine to alanine do not affect the proteolytic activity of this enzyme significantly, probably because the structural changes can be accommodated during global folding of the mutated protease (*10*).

Nitric oxide (NO), a fast-acting free radical, plays a significant role in nonspecific immunity of the host (*14*). It is generated by a variety of cell types under physiological and pathological conditions including bacterial, fungal, and viral infections. Low-level iNOS expression and NO production have been reported in human macrophages infected with HIV-1 (*15*). The oxidized form of NO, nitrosonium ion, has a high propensity to react with the thiol groups of cysteine residues. It also reacts with OH groups of tyrosine residues and secondary amines of lysine and arginine, but with 1000-fold lower reactivity than that toward the −SH group of cysteine (*16*). S-Nitrosation of proteins yields nitrosothiol adducts and results in different biological effects (*17*). However, as NO is unstable under physiological conditions, many of these effects are thought to be initiated by NO derived from *S*-nitrosothiols (*18*, *19*). These findings, along with the recent report that NO-treated virus loses its replication efficiency in monocyte-derived macrophages, have led us to hypothesize that nitrosonium ion (NO^+) may react with the cysteine residues of HIV-PR and may affect its proteolytic activity. Here, we show that the cysteine residues of HIV-PR can be reversibly modified by NO, forming nitrosothiol adducts with concomitant loss of its activity.

MATERIALS AND METHODS

Materials. iNOS, expressed and purified from bacteria, was a kind gift from Dr. Steven Gross of the Department of

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[1] Abbreviations: HIV-PR, HIV-1 protease; NO, nitric oxide; iNOS, inducible nitric oxide synthase; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; NEM, *N*-ethylmaleimide; ESI-MS, electrospray ionization-mass spectrometry.

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Pharmacology, Weill Medical College of Cornell University, New York, NY. Sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), isopropyl β-D-thiogalactopyranoside, and protease inhibitors were purchased from Sigma (St. Louis, MO). SNP and SNAP solutions were prepared fresh each time immediately before use. SNAP was dissolved in DMSO and then diluted in reaction buffer to achieve the desired concentration. Luria broth was purchased from Boehringer Mannheim (Indianapolis, IN). Ritonavir (a kind gift from Dr. Sumesh Kaushal, NIH) was prepared in ethanol as a 50 μM stock solution and stored at 4 °C. All other chemicals were of analytical grade.

Expression and Purification of HIV-1 Protease. The synthetic cDNA of HIV-PR (a kind gift from Y.-S. Edmond Cheng) was expressed as a 156 amino acid long precursor protein in BL21(DE3)pLysS cells (Novagen, Inc., Madison, WI) from a modified pET3a expression vector, pET3AM (*20*). The protease was purified from inclusion bodies by selective extraction and membrane fractionation using 50% acetic acid as described by Gustafson et al. with slight modifications (*21*). The renatured protease was dialyzed against phosphate buffer (100 mM at pH 6.7) containing 10% glycerol and stored with 40% glycerol at −20 °C for long-term use. Original pilot experiments used HIV-PR supplied by the NIH AIDS Research and Reference Program.

Protease Assay. The HIV-1 protease assay was performed according to Matayoshi et al., with slight modifications (*22*). The fluorogenic substrate R-E-(EDANS)-S-Q-N-Y-P-I-V-Q-K-(DABCYL)-R was obtained from Molecular Probes, Inc. (Eugene, OR). The assays were performed at 37 °C in 100 μL of acetate (0.1 M, pH 4.5) or phosphate (0.1 M, pH 6.7) buffer containing NaCl 1.0 M, DMSO 10%, EDTA 1.0 mM, and BSA 0.1 mg/mL in standard 96-well FluorNunc plates. The concentrations of enzyme, 50 nM, and substrate, 4.0 μM, were kept constant in all experiments. Samples were treated as indicated, and the activity was measured for 5 min. The reaction mixtures were excited at 360 nm, and fluorescence was measured at 535 nm in a fluorescence microplate reader (Spectrafluor, TECAN, Research Triangle Park, NC). The assay was linear over 5 min. In a typical experiment, about 10 000 fluorescence units were generated upon addition of substrate to enzyme alone. The change in fluorescence was expressed either as percent activity or as relative fluorescence intensity. Each data point is an average of three experiments, each performed in triplicate.

HIV-PR was treated with different concentrations of SNP and SNAP for 5 min at room temperature and then assayed for activity. Exposure of HIV-PR to enzymatically synthesized NO was achieved by adding HIV-PR in iNOS buffer (Tris-HCl, 10 mM at pH 7.0; L-arginine, 1 mM; NADPH, 1 mM) and different concentrations of purified iNOS and incubating the reaction for 10 min at 37 °C. Immediately following the incubation, an equal volume of protease assay buffer (2×) was added to the reaction mixture, and the activity of HIV-PR was measured. The amount of nitrite generated was measured by the Griess reaction (*23*).

Mass Spectrometry. Electrospray ionization-mass spectrometry (ESI-MS) of HIV-PR was performed using a Quattro II Triple Quadrupole Mass Spectrometer (Micromass Ltd., Wythenshawe, U.K.). HIV-PR, 5 μM, was treated with a 100 μM sample of an NO-saturated solution in 5 mM ammonium acetate buffer at pH 4.9 for 5 min at room temperature. For NEM studies, 5 μM HIV-PR in ammonium acetate buffer, 20 mM, was modified with 1 mM NEM for 5 min at room temperature. Combined modification of HIV-PR with NO and NEM was achieved by allowing the protease to react with 1 mM NEM and 100 μM NO solution for 10 min. The treated protease was then mixed with $CH_3OH/H_2O/CH_3COOH$ (50:50:2.0, v/v/v) and infused into the electrospray source of the mass spectrometer. The molecular masses of the protease and its modified forms were obtained from positive ion electrospray spectra using a maximum entropy deconvolution routine. Conditions for the identification of NO-modified species on proteins using ESI-MS have previously been described (*24*).

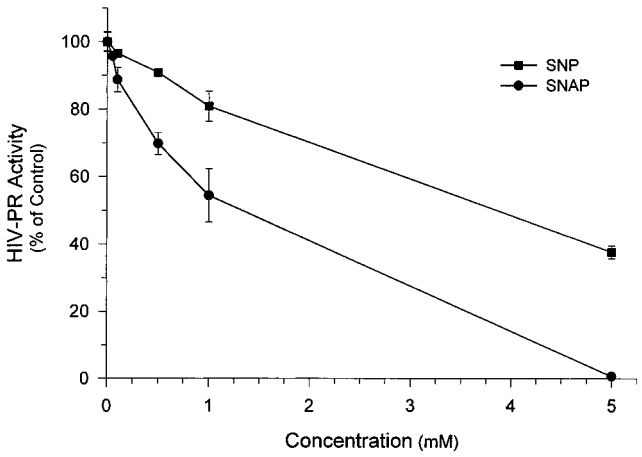

FIGURE 1: Inactivation of HIV-PR by SNP and SNAP. HIV-PR was treated with different concentrations of SNP and SNAP for 5 min at room temperature and then assayed for protease activity using a fluorogenic substrate. The obtained fluorescence at each concentration of SNP and SNAP was compared with the control fluorescence and is represented as a percent of control activity.

RESULTS

Inhibition of HIV-PR by NO-Related Species. HIV-PR was expressed in BL21(DE3)pLysS cells and purified from inclusion bodies by selective extraction and membrane fractionation using 50% acetic acid. The protein was refolded and its activity examined. The processed form of recombinant protease was obtained at greater than 90% purity as determined by SDS−PAGE analysis and Coomassie blue staining. The unprocessed form of HIV-PR (156 aa) was copurified with the processed form (99 aa) at a ratio of 10:90 (data not shown). The yield of the recombinant protease from 1 L of bacterial culture was approximately 3.0 mg.

To determine the effect of NO on HIV-PR activity, the enzyme was preincubated in the presence or absence of various concentrations of SNP or SNAP for 5 min at room temperature. As seen in Figure 1, preincubation of HIV-PR at low concentrations of SNP resulted in a nominal decrease in its activity, whereas 62.4 ± 0.9% inhibition was achieved with 5 mM SNP. When treated with SNAP 5.0 mM, protease activity was completely abolished within 5 min of incubation.

To analyze the effect of exposure time of HIV-PR to NO, kinetic studies were performed. The enzyme was treated with SNP (30 μM) or SNAP (100 μM) for different periods of time, and its activity was measured. As seen in Figure 2, inhibition was biphasic with an early rapid effect and a much lesser effect at later times. Untreated enzyme demonstrated

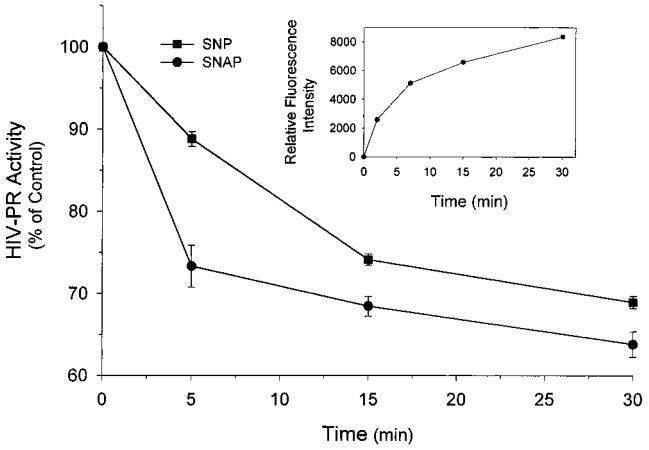

FIGURE 2: Time course of inactivation of HIV-PR by SNP and SNAP. HIV-PR was treated with SNP (30 μM) and SNAP (100 μM) at room temperature for the indicated times prior to assay. The inset shows the activity of untreated HIV-PR assayed under identical conditions.

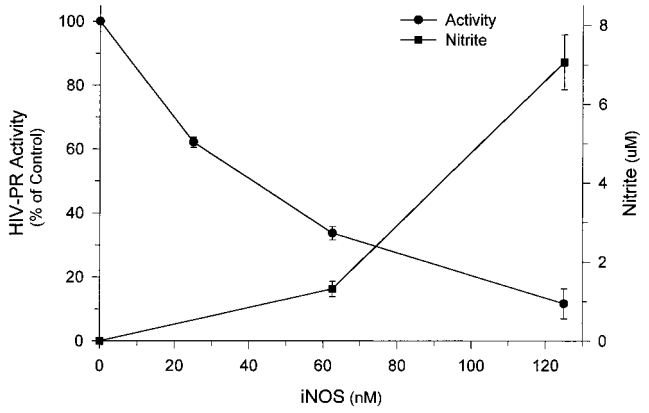

FIGURE 3: Inactivation of HIV-PR by iNOS. iNOS was mixed with HIV-PR in a buffer containing all of the cofactors necessary for iNOS activity (see Materials and Methods). After 10 min, protease activity and nitrite concentration were measured.

no loss in its activity during the course of incubation (insert). These data indicate that most of the inhibition occurred in the first 10 min and suggest a single target.

In an attempt to mimic a more physiological setting, iNOS was used to generate NO from L-arginine. HIV-PR was added to a reaction mixture which contained its cofactors and different concentrations of iNOS. After 10 min, the reaction mixture was acidified, and HIV-PR activity was measured. To determine total nitrite production in the reaction mixture, samples were treated first with nitrate reductase and then with a lactate dehydrogenase/pyruvate mixture. The amount of nitrite was estimated by the Griess reaction. Total nitrite obtained with 62.5 and 125 nM iNOS was 1.3 $\pm$ 0.3 and 7.0 $\pm$ 1.2 μM, respectively, after 10 min. Nitrite produced by 31.25 nM iNOS could not be detected. Figure 3 demonstrates that NO generated in vitro by iNOS inhibited HIV-PR in a concentration-dependent manner. These data clearly indicate that HIV-PR can be inhibited by NO generated in situ by iNOS or NO donors.

Mechanism of Inhibition. Ritonavir is a known competitive peptide inhibitor of HIV-PR (*25*). To determine whether the inhibitory effect of NO on HIV-PR occurs due to modification at a similar or distinct site of action, we analyzed the inactivation of HIV-PR in the presence or absence of

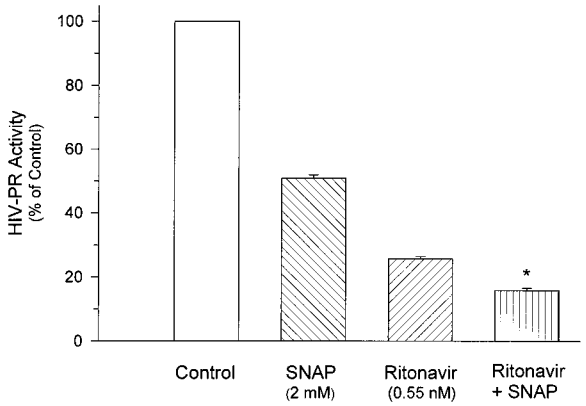

FIGURE 4: Inhibition of HIV-PR by SNAP and ritonavir. HIV-PR was treated with SNAP (2.0 mM) or ritonavir (0.55 nM) or sequentially with SNAP and ritonavir for 5 min at room temperature as described under Materials and Methods. The asterisk indicates a significant difference between ritonavir alone and ritonavir and SNAP using a paired *t*-test.

ritonavir in conjunction with SNAP. The effect of ritonavir alone and on NO-pretreated HIV-PR is shown in Figure 4. In the case of combined treatment, enzyme was pretreated with SNAP for 5 min at room temperature and then ritonavir was added prior to the assay. A combination of SNAP and ritonavir inhibited the enzyme by 84.1 $\pm$ 1.8% while, individually, 74.3 $\pm$ 2.0% and 49.1 $\pm$ 3.0% inhibition was achieved with ritonavir and SNAP, respectively. These results suggest that the inhibition of HIV-PR by ritonavir and SNAP are attained via two different mechanisms.

To determine whether NO-inactivated HIV-PR could have its activity restored, we added reducing agents to SNAP-pretreated HIV-PR (Figure 5A). Treatment of HIV-PR with SNAP alone inhibited its activity by 75%. When incubated in the presence of DTT or β-mercaptoethanol, most of the HIV-PR activity was restored. Reduced glutathione had no effect on the restoration of activity of NO-modified HIV-PR. To determine whether this reversal of activity by thiol agents was time-dependent, we added thiol agents to SNAP-inactivated HIV-PR and measured activity over 1 h. We found (Figure 5B) a clear time-dependent reversal of SNAP inhibition by both dithiothreitol and β-mercaptoethanol. Of interest is that reduced glutathione was unable to reverse this inhibition, suggesting that, at least in vitro, strong reducing agents are required for reversal. These data suggest that NO modifies a cysteine thiol on HIV-PR.

Formation of S-Nitrosothiols on HIV-PR. To determine whether nitric oxide modified the cysteine residues of HIV-PR, the enzyme was treated with a 100 μM sample of an NO-saturated solution for 5 min at room temperature and subjected to eletrospray ionization-mass spectrometry (*24*). Molecular masses of unmodified and NO-modified species of HIV-PR were obtained from a maximum entropy deconvolution routine. In solution, the protease was found to exist in both monomeric (10 779 Da) and dimeric (21 556 Da) forms (Figure 6A). Upon NO (mass = 30 Da) treatment, the mass of the monomer shifted by 30 Da (new mass of 10 809 Da) and 60 Da (new mass of 10 839 Da), which corresponds to the addition of one and two NO molecules to the monomer, respectively (Figure 6B). In addition, two new dimers were observed with masses of 21 616 and 21 676 Da (Figure 6B). These differences in mass (60 and 120 Da)

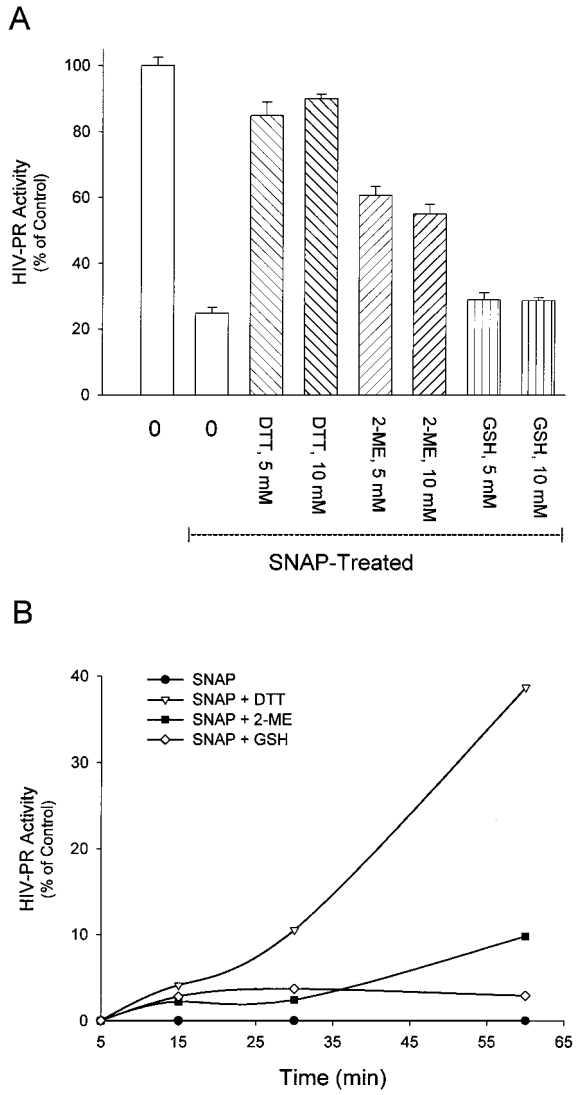

FIGURE 5: Reversibility of protease inhibition. (A) HIV-PR was treated with 2.0 mM SNAP for 5 min at room temperature, and the activity was assayed. In separate reactions, SNAP-treated enzyme was incubated for 1 h with the identical concentrations of DTT, β-mercaptoethanol (2-ME), and reduced glutathione (GSH). (B) SNAP-treated HIV-PR was incubated with the indicated reducing agents (10 mM each) immediately after SNAP treatment, and protease activity was measured over 1 h. Control refers to basal activities of SNAP-treated enzyme which were 24, 37, 45, and 50% at 5, 15, 30, and 60 min, respectively.

correspond to the binding of two and four NO molecules to the dimeric form of the protease, respectively. The existence of two cysteine residues per monomer and the appearance of two NO-modified forms of monomer, and the presence of four cysteine residues per dimer and the appearance of two doubly NO-modified forms of dimer strongly suggest that cysteine residues of the protease are targets of nitric oxide. Furthermore, no tyrosine nitration (Tyr-NO_2) occurred as this would yield an increased mass of 45 Da, which was not observed.

To confirm that modification of the protease by nitric oxide is due to S-nitrosation of its cysteine residues, we performed a competition experiment with *N*-ethylmalemide (NEM, mass = 125 Da). Cysteine residues were modified by incubating the enzyme for 1 h with NEM. On complete modification, the monomer and dimer of HIV-PR acquired new masses increased by 250 and 500 Da, respectively, which were due to the addition of two molecules of NEM to the monomer (new mass = 11 029 Da) and four molecules of NEM to the dimer (new mass = 22 058 Da) (Figure 7A). This confirms the specificity of NEM and our ability to measure it. When HIV-PR was allowed to react simultaneously with NO and NEM for 5 min, multiple species corresponding to the monomeric and dimeric forms were generated, modified by either NO or NEM or by both (Figure 7B,C). The number of new peaks, which appeared during simultaneous treatment, was limited by the number of cysteine residues present in the protease, modified by either NO or NEM. Addition of two NO molecules to the monomer prevented NEM molecules from binding to it and vice versa. Similarly, further addition of NEM molecules to the dimer could not be achieved on a fully NO-modified species and vice versa. These data confirm that NO binds directly to Cys67 and Cys95 of HIV-PR to form *S*-nitrosothiols.

DISCUSSION

Cysteine-containing proteins are susceptible to modification by NO species. Many cytosolic and membrane-bound proteins and enzymes have been shown to be regulated by S-nitrosation at active sites or at regulatory sites (*17*). Viral proteins and proteins of other pathogenic organisms are also S-nitrosated in cells due to a surge of NO production generated in response to infection (*14*). In the case of HIV-1 infection, monocytes/macrophages play a crucial role in both latency and diffusion of the infection. These cells produce a significant amount (2−5 μM) of NO during HIV-1 infection (*26*). Here we present evidence indicating that in vitro, HIV-1 protease can be inhibited by nitric oxide through direct modification of its cysteine residues. Using SNP and SNAP as nitric oxide donors, and iNOS to generate NO in situ, we have shown that significant inhibition of HIV-PR activity can be achieved at the same time at which we detect S-nitrosation of cysteine residues. These data, coupled with our ability to reverse the inhibition with reducing agents, strongly suggest that this inhibition is caused by direct modification of cysteine residues.

The kinetics and amount of NO generated from SNP and SNAP are different. While the SNAP-treated enzyme lost all of its activity within 5 min, only 70% inactivation of HIV-PR could be seen with SNP. The difference in inhibition of HIV-1 protease by SNP and SNAP can be explained by the amount of NO^+ (nitrosonium ion) generated by these donors. We performed the Griess assay to measure the amount of nitrite produced from 5 mM SNP and SNAP and found 1.1 and 9.7 μM, generated in 10 min, respectively. Since the total NO generation from these congeners is a mixture of several species (NO, NO^+, NO^-), the amount of NO^+ in solution is even less. Thus, although we require seemingly excessive amounts of NO-generating compounds to inhibit the HIV-PR in vitro, the actual amount of NO species generated in 10 min is far less than that of the added donor. The inhibition of HIV-PR by NO generated in vitro by iNOS complemented the results of SNAP and SNP. Approximately 90% inhibition of the protease could be achieved using 125 nM of purified iNOS. The total nitrite measured in the reaction was 7.0 μM, which is similar to the amount obtained from 5 mM SNAP.

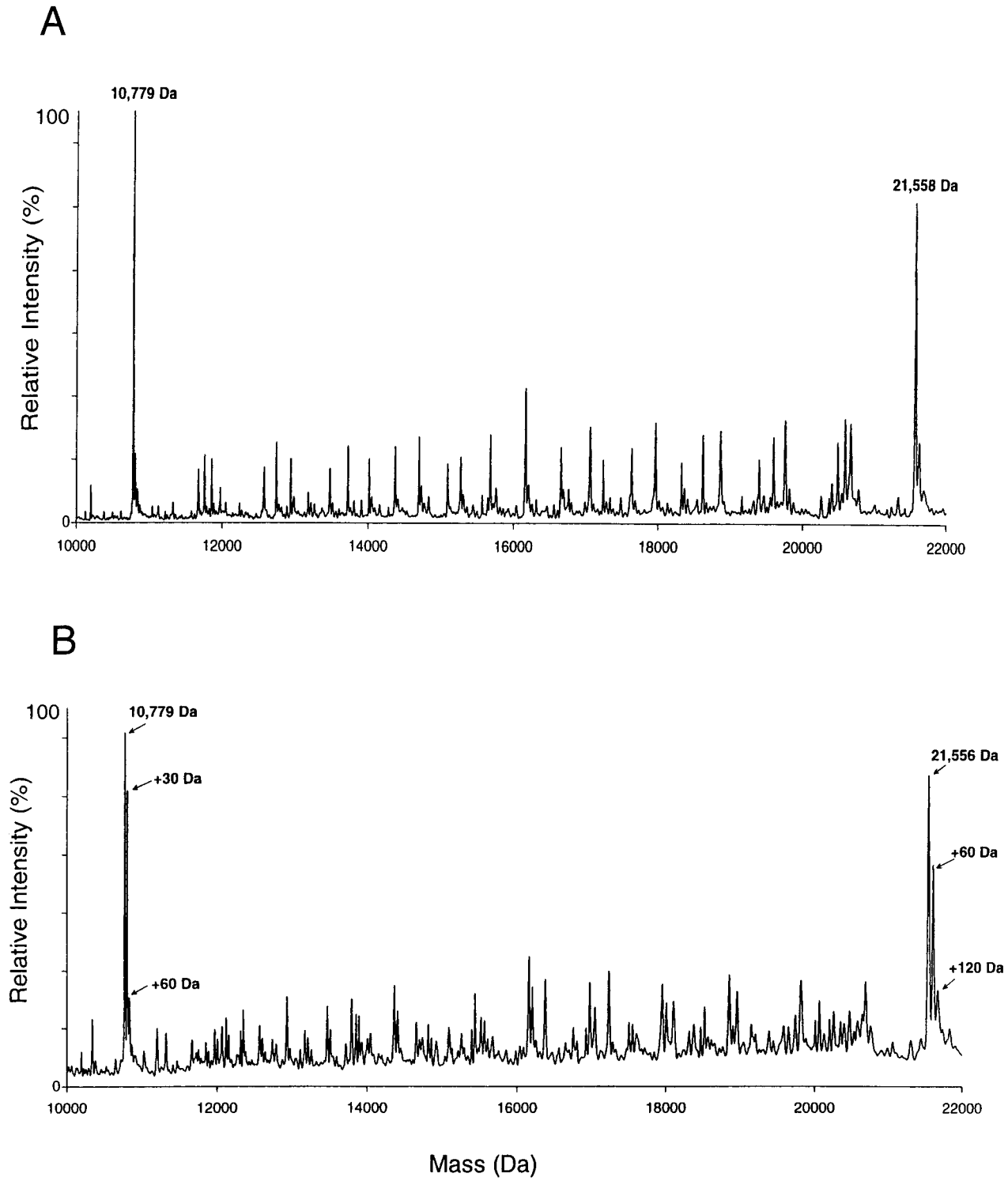

FIGURE 6: ESI-MS of NO-treated HIV-PR. HIV-PR (2 μM) was treated without (A) or with (B) an NO-gas saturated solution (100 μM) for 5 min at room temperature prior to infusion into the electrospray source after mixing with $CH_3OH/CH_3COOH/H_2O$ (50:50:2.5, v/v/v). Source temperature and cone voltage were 60 °C and 30 V, respectively. Observed molecular masses (±2 Da) of the unmodified (A) monomer and dimer and different NO-modified (B) species are labeled.

Our finding that inhibition of HIV-PR by NO is reversed by thiol agents not only supports cysteine thiols as a target but also suggests that perhaps in cells, redox regulation of HIV-PR occurs. It is possible that stressed cells, where endogenous thiols are depleted, possess a more oxidizing environment and may be more likely to utilize NO as an antiviral agent. In contrast, in healthy cells, NO would be less able to attack cysteine residues. The subcellular localization of the interaction between HIV-PR and NO would likely occur at the site of HIV-PR action. Perhaps the high lipid-solubility of NO allows penetration into vesicles where virus formation takes place.

In a recent communication, Persichini et al. have shown that HIV-PR could be inactivated by the NO donor (*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3) (*9*). However, other than qualitative spectrometry, no physical analysis was performed to determine if the HIV-PR was modified by NO generated by NOR-3. Using electrospray

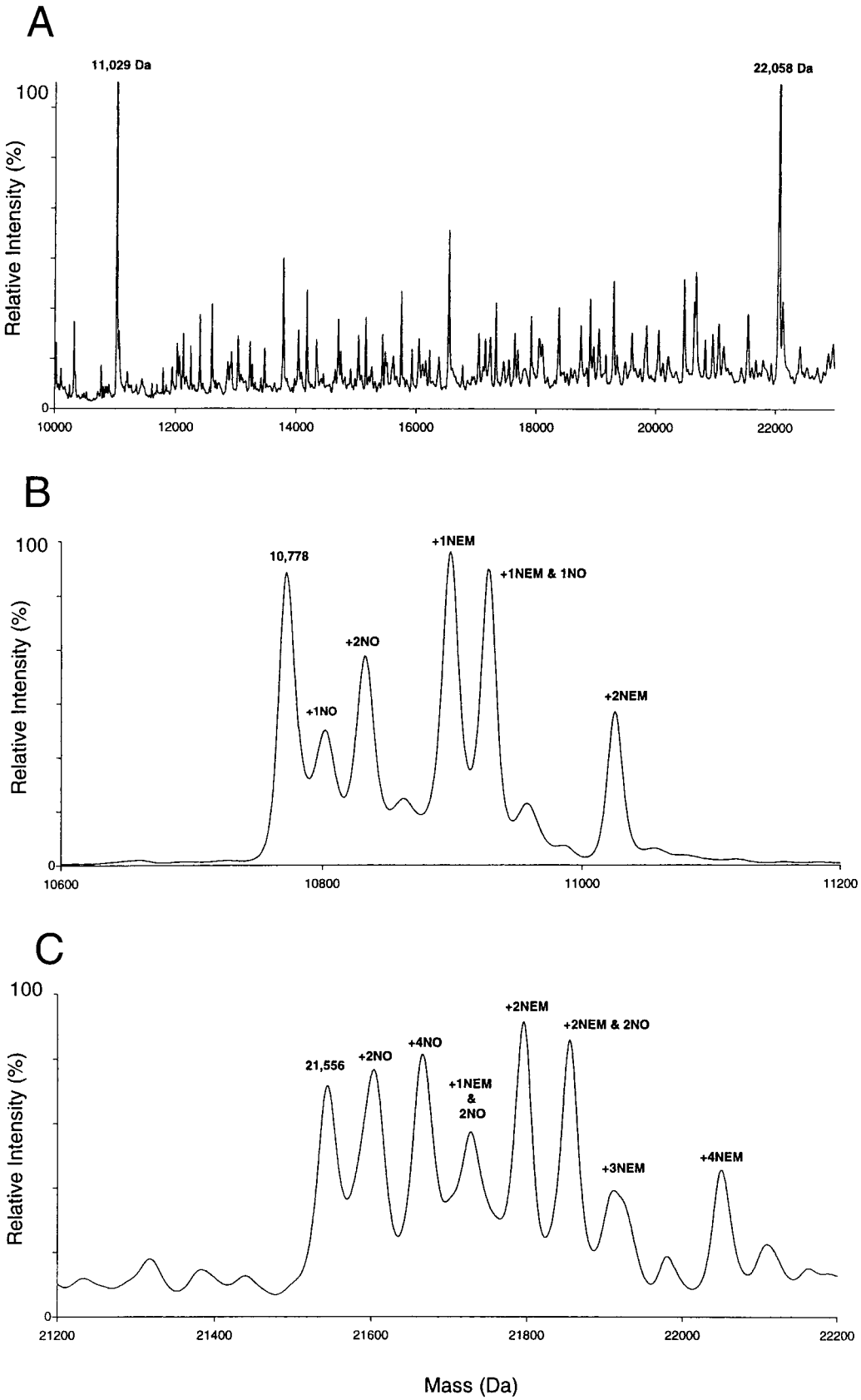

FIGURE 7: NO and NEM compete for the same sites on HIV-PR. HIV-PR (2 μM) was treated with NEM (1.0 mM) (A) alone or in combination with both NEM (1.0 mM) and an NO-gas saturated solution (100 μM) (B and C) for 5 min at room temperature and then subjected to ESI-MS. Those peaks corresponding to a mass increased by 30 Da are labeled as +NO, and those corresponding to a mass increased by 125 Da are labeled as +NEM. Panels B and C are expanded ranges of the monomer and dimer, respectively.

ionization-mass spectrometry, we were able to identify the NO-modified species of HIV-PR, thereby providing direct evidence of its modification. In solution, monomer and dimer of HIV-PR exist in equilibrium. When treated with NO gas,

modified species of both monomer and dimer are seen, their masses differing by multiples of the mass of NO. The number of modified species of HIV-PR coincides with the number of cysteine residues present in each monomer. Complete modification of HIV-PR by the thiol-binding agent NEM prevented modification by NO, confirming that NO targeted the cysteine residues. Simultaneous treatment of HIV-PR with NO and NEM yielded different intermediate species modified by both NO and NEM along with the appearance of completely modified forms. These data, supported by competition experiments with NEM, clearly establish that S-nitrosation of the HIV-PR occurs at cysteine residues. Simultaneous S-nitrosation and loss of activity of HIV-PR suggests that Cys67 and Cys95 have profound effects on the activity of HIV-PR. Incomplete modification of the enzyme by NO would explain the residual activity of the HIV-PR after SNP or SNAP treatment. Stability studies revealed that the dimer interface of HIV-PR contributes almost 75% of the total Gibbs energy of association (*27*). Modification of Cys95 by NO may lead to a significant perturbation of this energy state and thereby slightly disrupt the dimer with concomitant loss of proteolytic activity. Further studies are required to elucidate individual contributions of each modification to the inhibition of HIV-PR.

Cysteine modification seems to be a common way the activities of cellular proteins are regulated, either under normal physiological conditions or under pathological situations, and it is becoming evident that NO is the molecule of choice for cysteine residue modification in cells (*17*, *28*). Nitric oxide exerts microbiostatic and microbicidal activity against a broad range of pathogens (*29*, *30*). Compelling evidence comes from Hori et al., who have shown that HIV-1 replication in monocyte-derived macrophages is inhibited by NO produced by primary astrocytes (*15*). Our findings, that HIV-1 protease is inactivated by NO (and therefore is unable to generate the processed form of viral reverse transcriptase from polyprotein), explains the lack of viral replication in those cells. That HIV-1 protease is susceptible to inactivation by NO, together with the fact that NO produced by dendritic cells impairs the T cell response (*31*), suggests that NO production may be an important mediator of resistance of dendritic cells to HIV-1 infection.

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