Regulation of HIV-1 Protease Activity through Cysteine Modification

David A. Davis,[‡] Karen Dorsey,[‡] Paul T. Wingfield,[§] Stephen J. Stahl,[§] Joshua Kaufman,[§] Henry M. Fales,[||] and Rodney L. Levine*,[‡]

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-0320, Protein Expression Laboratory, Office of the Director, Bethesda, Maryland 20892-2775, and Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892-1676

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ABSTRACT: The homodimeric protease of the human immunodeficiency virus 1 contains two cysteine residues per monomer which are highly conserved among viral isolates. However, these cysteine residues are not essential for catalytic activity which raises the question of why they are conserved. We have found previously that these cysteine residues are unusually susceptible to oxidation by metal ions, and this results in inhibition of protease activity. Recombinant protease mutants (C67A, C95A, and the double mutant C67A,C95A) were prepared to assess the possible role of these cysteines in redox regulation of the enzyme. Mixed disulfides were formed between the cysteine residues of the enzymes and low molecular weight thiols. Enzyme activity was lost when a mixed disulfide was formed between 5,5′-dithiobis(2-nitrobenzoic acid) and cysteine 95, while the same mixed disulfide at cysteine 67 reduced activity by 50%. This effect was reversible as normal activity could be restored when the enzyme was treated with dithiothreitol. The cysteines could also be modified with the common cellular thiol glutathione. Modification with glutathione was verified by mass spectrometry of the protein peaks obtained from HPLC separation. Glutathiolation of cysteine 95 abolished activity whereas modification at cysteine 67 increased the k_{cat} by more than 2-fold with no effect on K_m. In addition, glutathiolation at cysteine 67 markedly stabilized the enzyme activity presumably by reducing autoproteolysis. These results demonstrate one possible mechanism for regulation of the HIV-1 protease through cysteine modification and identify additional targets for affecting protease activity other than the active site.

Maturation of the human immunodeficiency virus type 1 (HIV-1)[1] requires limited proteolytic processing of the *gag* and *gag-pol* polyproteins during virion budding (Oroszlan & Luftig, 1990). These polyproteins, which contain the enzymes and structural proteins of the mature virus, are processed by a viral-encoded protease which itself is contained within the *gag-pol* polyprotein. Studies indicate that the protease can carry out its own excision from the polyprotein (Debouck et al., 1987; Louis et al., 1994). Overexpression of protease activity in infected cells either by expression of a genetically linked protease dimer or by mutation of the frameshift signal in the *gag-pol* mRNA results in loss of particle formation and cell death (Krausslich, 1991, 1992; Mergener et al., 1992). However, partial inhibition of the more active single-chain protease, generated by encoding a single protein consisting of two sequentially encoded subunits, can restore viral particle formation and prevent cell toxicity. Additionally, partial inhibition of HIV-1 protease activity in infected cells can dramatically alter the infectivity of the viral particles produced (Kaplan et al., 1993). Although the proteolytic processing leading to viral maturation is a sensitive process in the viral life cycle (Katz & Skalka, 1994), it is not known how this process is regulated during viral replication.

Each monomer in the homodimeric HIV-1 protease contains two cysteines which are highly conserved among viral isolates (Myers et al., 1993). Although the cysteine residues are not required for protease activity, the conserved nature of these residues suggests they may be important for the normal function and/or regulation of the protease in HIV-1-infected cells. Other proteins, including papilloma virus E2 protein, adenovirus protease, and citrate synthase, contain non-active-site cysteines that appear to serve regulatory roles (Donald et al., 1991; Tamai et al., 1991; Grasser et al., 1992; McBride et al., 1992; Webster et al., 1993). The HIV-1 protease is sensitive to agents which react with sulfhydryls such as copper, *N*-ethylmalemide, iodoacetamide, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (Meek et al., 1989; Karlstrom & Levine, 1991; Karlstrom et al., 1993). Although sulfhydryl reagents inhibit protease activity, it is not clear which of the cysteine residues contribute to this effect. Studies with a protein-extended form of the protease provided evidence that derivatization of the surface-exposed residue, cysteine 67, with DTNB was sufficient to inhibit the protease (Karlstrom & Levine, 1991). Subsequently, using a 17 amino acid model peptide spanning the region around cysteine 67, it was found that this cysteine is more reactive toward DTNB under native conditions than

* Address correspondence to this author at NIH, Buildingg 3, Room 106, 3 Center Dr., MSC 0320, Bethesda, MD 20892-0320. Telephone: (301) 496-2310. Fax: (301) 496-0599. E-mail: rlevine@nih.gov.

[‡] Laboratory of Biochemistry, National Heart Lung, and Blood Institute.

[§] Protein Expression Laboratory.

[||] Laboratory of Biophysical Chemistry, Natioanl Heart Lung, and Blood Institute.

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[1] Abbreviations: AIDS, acquired immune deficiency syndrome; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; *gag,* group-specific antigen; HIV-1, human immunodeficiency virus type 1; RP-HPLC, reverse-phase high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; *pol,* polymerase; TFA, trifluoroacetic acid; TNB, 5-thio-nitrobenzoic acid.

when denatured (D'Ettorre & Levine, 1994). Reactivity was attributed to the neighboring amino acids which promote ionization of the cysteine residue. This increased reactivity of cysteine 67 toward DTNB has also been observed for the mutant enzyme C95A (unpublished data). In this study, we used three recombinant protease mutants (C67A, C95A, and C67A,C95A) to study the role of each cysteine in protease function. We also investigated the effect of S-thiolation on protease activity with the cellular thiol, glutathione.

MATERIALS AND METHODS

Protein Expression and Purification. HIV-1 protease (strain HXB2) was produced in *Escherichia coli* (Cheng et al., 1990) using the expression vector pET11a and the host bacterial strain BL21 (DE3) (Studier et al., 1990). The cysteine to alanine mutations were introduced using the polymerase chain reaction (Mullis et al., 1986; Higuchi et al., 1988).

Cells were grown in minimal media at 37 °C, 30% pO_2, pH 7.0, in a 2-L Braun model MD fermentor. When the cells reached an optical density of approximately 5 at 600 nm, protein expression was induced with 2 mM IPTG at 32 °C for 4 h. The cell pellet (approximately 25 g wet weight) was suspended in 5 volumes of buffer which contained 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM benzamidine hydrochloride, and 5 mM dithiothreitol (DTT). Cells were lysed using a French press and clarified by centrifugation at 20000*g* for 45 min. The insoluble HIV-1 protease was washed by resuspension in 0.5% Triton X-100 and 1 M urea; centrifugation was as above for 30 min. This step was repeated twice and the final pellet solubilized with 30 mL of 50 mM Tris-HCl, pH 7.5, containing 8 M guanidine hydrochloride, and 5 mM DTT. After clarification of the extract by centrifugation it was applied to a Superdex 200 gel filtration column (6 cm x 60 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, 4 M guanidine hydrochloride, 1 mM EDTA, and 2 mM DTT. The column was eluted at 4 mL/min, and fractions containing protease were pooled and concentrated by ultrafiltration to 40−45 mL. The concentrate was applied to a Superdex 75 gel filtration column (6.0 cm × 60cm), equilibrated, and eluted as above. The peak fractions containing protease were pooled and stored in aliquots (approximately 1 mg/mL) at −80 °C. Aliquots (10 mL) were applied to a Source 15 reverse phase column (1.0 cm × 10 cm; Pharmacia) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water. The protein was eluted at 5 mL/min using a linear gradient (3 column volumes) of 35−100% acetonitrile. The protein was refolded as described (Karlstrom & Levine, 1991) and stored in 20 mM HCl, pH 1.6, at −70 °C until further use. Proteases containing the cysteine to alanine mutations were processed as described above except they were labeled with ^{15}N for use in NMR studies reported elsewhere (Yamazaki et al., 1994).

HIV-1 Protease Assay. Protease activity was determined as described (Boutelje et al., 1990). For assays with the purified DTNB-modified enzymes, each enzyme (2.5 μL of 0.1−0.3 μM) was added to 5 μL of 2× sodium phosphate buffer pH 7.1, 20% glycerol, 10% ethylene glycol, and 0.2 mg/mL BSA with or without 10 mM DTT and incubated for 10 min. The pH was then brought to 5.5 with the addition of 2.5 μL of 0.4 M acetic acid and assayed for 30−60 min. For kinetic experiments, the assay was started with the addition of enzyme to assay buffer containing substrate and 1 M KCl. High salt was included in the assay because it gave more consistent results in activity. Substrate concentration was varied from 0.5 to 5 mM, and the kinetic parameters were obtained by fitting the data to the Michaelis−Menten equation using the program Origin (MicroCal Software, Northhampton, MA). For analysis of the reaction products in the kinetic experiments, the more sensitive AQC method for detecting the peptide products was used (Davis et al., 1995). Determination of protease stability was done by incubating each enzyme at 1 μM in buffer (without substrate) containing 150 mM sodium acetate, pH 5.5, 1 M KCl, 10% glycerol, 5% ethylene glycol, and 1 mM Na_2-EDTA. At 10 min time intervals, 10 μL aliquots were analyzed for activity in a 6 min assay.

Derivatization of Cysteine Residues of the HIV-1 Protease with DTNB. Protease (400 μL of 6−50 μM solution) was treated with DTNB and incubated for 10 min in buffer, final concentration 150 mM sodium phosphate, pH 6.2, containing 10% glycerol and 5% ethylene glycerol. Reagent was removed by dialysis with 6000−8000 MW cutoff tubing (Spectrum Medical Industries, Houston, TX) against deionized water adjusted to pH 1.6 with HCl. The extent of cysteine modification with DTNB and enzyme concentrations were determined by analyzing a fraction of the enzyme on RP-HPLC with detection at 328 nm for the TNB chromophore and 275 nm for protein. The retention times for the modified C67A (TNB-C95) and C95A (TNB-C67) increased by approximately 0.5 min.

Derivatization of Cysteine Residues of the HIV-1 Protease with Glutathione. Protease (400 μL of 20−75 μM in deionized water, pH 1.65, with HCl) was added to 400 μL of 300 mM sodium phosphate buffer, pH 7.1, containing 20% glycerol and 10% ethylene glycol with or without 0.1−50 mM oxidized glutathione (Sigma, St. Louis, MO). Reactions were terminated by the addition of 25 μL of 50% TFA, followed by the addition of solid guanidine to 6 M. The reaction mixture was then analyzed and separated by RP-HPLC. Solvent A was deionized water containing 0.05% TFA, and solvent B was acetonitrile containing 0.05% TFA. For separation of the protease glutathiolated at cysteine 67, the gradient was increased from 0 to 30% solvent B at 3%/min followed by an increase from 30% to 60% solvent B at 1.5%/min, and then ramped from 60% to 100% solvent B at 20%/min. The protease was detected by following the elution at both 205 and 275 nm. The gradient for separation of glutathiolated cysteine 95 from the unmodified form consisted of a linear increase from 0 to 40% solvent B at 4%/min followed by an increase from 40% to 53% at 0.5%/min, and then ramped from 53% to 100% solvent B at 20%/min. The peaks corresponding to the modified and unmodified forms of the protease were collected and refolded (Karlstrom & Levine, 1991). Protease concentrations for the RP-HPLC repurified enzymes were calculated from the molar absorptivity ($\epsilon = 12\,300$) or by amino acid analysis (Jones & Gilligan, 1983). These preparations were also reanalyzed by RP-HPLC to verify that measurable autoproteolysis did not occur during refolding.

Determination of Protease Modification by Mass Spectrometry. HIV-1 protease samples from RP-HPLC were analyzed by electrospray mass spectrometry to verify modification with glutathione. Samples were evaporated and

Table 1: Comparison of Kinetic Parameters for the Wild-Type and Mutant HIV-1 Proteases

HIV-1 protease[a]	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
wild type	0.9 ± 0.2	15 ± 2	16.7
C67A	1.0 ± 0.1	16 ± 1	16.0
C95A	0.9 ± 0.3	16 ± 2	17.8
C67A,C95A	0.6 ± 0.2	20 ± 2	33.3

[a] Each enzyme at 1 μM was assayed for 3 min in buffer containing 150 mM sodium acetate, pH 5.5, 10% glycerol, 5% ethylene glycol, 1 M KCl, and 1 mM EDTA. Each enzyme was assayed in at least two separate experiments with the substrate varied from 0.5 to 5 mM. Kinetic parameters $\pm$ SD were obtained from the program Origin as described under Materials and Methods.

reconstituted using 0−5% acetic acid in 50% aqueous methanol. Mass spectra were obtained using a Finnigan TSQ 700 equipped with a Finnigan electrospray attachment. Samples were admitted using a Harvard syringe pump at 1 μL/min with the nozzle at 4500−5500 V and the capillary at 200 °C. Three second scans covering *m/z* 200−2000 were obtained and deconvoluted using the programs provided by Finnigan. The expected mass for the wild-type protease is 10 776 Da while that for the single C→A mutants, which were produced as ^{15}N-labeled enzymes, is 10 871 Da.

RESULTS

Protein Expression and Purification. The HIV-1 protease was expressed as a 138 residue fusion protein (Cheng et al., 1990) which, under the fermentation conditions described, was 50−75% processed into mature protease, a 99 residue protein. The expression level of mature protease was in the range of 10−15% of total cellular protein. The denatured and inactive protease was purified by two rounds of gel filtration in the presence of guanidine hydrochloride and finally by RP-HPLC. As protein purification is under denaturing conditions, no autoproteolytic cleavage of the protein occurred. The protease could be refolded and activity obtained as described (Karlstrom & Levine, 1991).

The mutant proteases analyzed under high salt conditions (C67A, C95A, and C67A,C95) had kinetic properties similar to the wild-type enzyme (Table 1). However, an increase in k_{cat} was observed for the C67A,C95A enzyme. Small changes in the kinetic parameters have been observed for other HIV-1 proteases produced with mutations at the cysteine positions (Salto et al., 1994). Thus, replacement of the cysteines with alanines does not decrease catalytic activity, and the kinetic parameters are similar to the wild type.

Modification of Cysteine Residues with DTNB Reversibly Inhibits HIV-1 Protease Activity. It has been demonstrated that sulfhydryl reagents inhibit the activity of the wild-type protease (Meek et al., 1989; Karlstrom et al., 1993). In order to assess the role of each cysteine residue in inhibition of protease activity, we studied the effect of DTNB on three protease mutants, C67A, C95A, and C67A,C95A. Each enzyme was treated with DTNB at pH 6.2 followed by dialysis to remove the excess reagent. The modified enzymes were purified by RP-HPLC by collecting TNB-derivatized protease which could be detected at 328 nm (Sliwkowski & Levine, 1985). The enzyme modified at cysteine 95 with DTNB was completely inhibited whereas the enzyme modified at cysteine 67 retained approximately 50% of its activity (Figure 1). The effect of DTNB

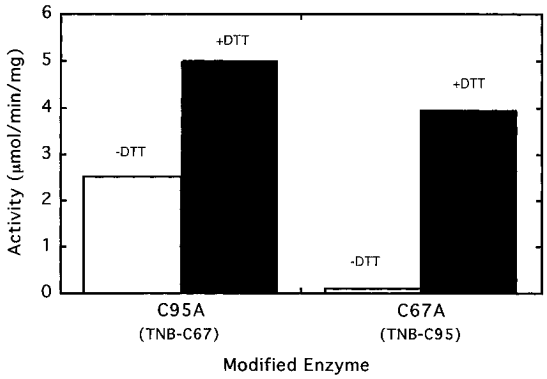

FIGURE 1: Effect of cysteine modification with DTNB on HIV-1 protease activity in the absence or presence of DTT. Each enzyme was modified with DTNB and assayed as described under Materials and Methods following purification of the modified enzyme by RP-HPLC. Each value is the average of two separate experiments with less than 4% variation in activity between experiments on C67A and less than 25% for C95A. (□) Control (untreated), (■) treated with 10 mM DTT.

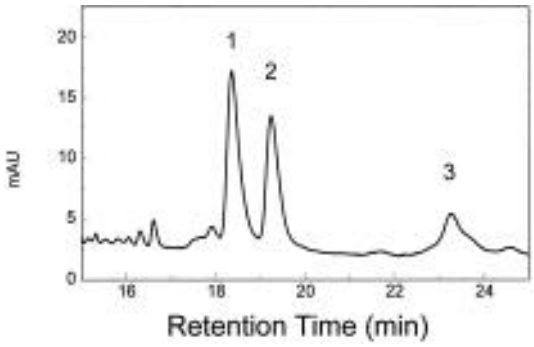

FIGURE 2: HPLC tracing at 276 nm for C67A HIV-1 protease after treating the enzyme for 30 min with 50 mM oxidized glutathione at pH 7.0. RP-HPLC was carried out as described under Materials and Methods. Peak 1 was identified by electrospray mass spectrometry as the glutathiolated form of the protease. Peak 2 was identified as the unmodified protease, and peak 3 was identified as the disulfide-linked dimer of the enzyme (see Figure 3 for mass analysis).

modification of the repurified enzymes paralleled that found with direct treatment of the protease with DTNB in the standard assay (data not shown). Incubation of each of the derivatized enzymes at pH 7.0 with 10 mM DTT restored the activity measured at pH 5.5, demonstrating that this type of inhibition by DTNB is reversible (Figure 1). This treatment also removed the TNB chromophore from the enzyme as determined by RP-HPLC (data not shown).

Modification of Cysteine Residues with Glutathione Reversibly Affects HIV-1 Protease Activity. The reversible inactivation of protease activity by DTNB led us to investigate the effect of a physiologically relevant sulfhydryl agent, glutathione. The single cysteine protease C67A (contains cysteine 95) was treated with 50 mM oxidized glutathione for 30 min and analyzed by RP-HPLC. In addition to the unmodified protease peak, two new peaks were obtained by HPLC (Figure 2). The first peak, which eluted prior to the unmodified protease (with a molecular mass of 10 871 Da, Figure 3, top panel), had a molecular mass of 11 178 Da as determined by electrospray mass spectrometry (Figure 3, middle panel). This molecular mass increase of 307 Da corresponds closely to the expected increase of 305 Da for the glutathiolated enzyme. The last peak constituted 16% of the total enzyme fraction and had a molecular mass of

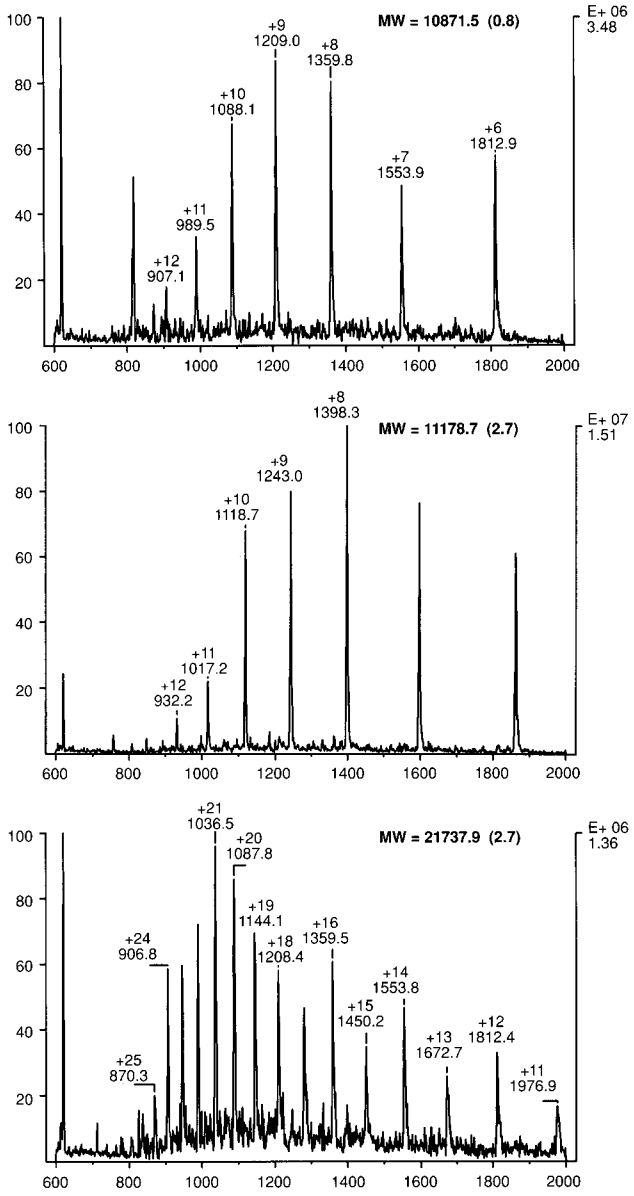

FIGURE 3: Identification of the molecular mass for the peaks obtained from HPLC analysis in Figure 2. Distribution of the charge states is shown in each panel. Deconvoluted masses with the standard deviations in parentheses are shown at the upper right of each panel. Top panel, molecular mass for HPLC peak 2 corresponding to the unmodified form of the protease; middle panel, molecular mass for HPLC peak 1 corresponding to the glutathiolated protease; bottom panel, molecular mass for HPLC peak 3 corresponding to the disulfide-linked dimer of the protease.

21 737 Da, which is very close to the expected molecular mass for the disulfide-linked dimer of the protease (21 740 Da) (Figure 3, bottom panel). Some monomer is also present in this fraction as revealed by the excess intensities of the +12 to +16 charge states of the monomer. Glutathione treatment of the C95A (contains cysteine 67) protease produced both the glutathiolated protease (molecular mass of 11 178 Da) and the disulfide-linked dimer (molecular mass of 21 746 Da) identified by mass spectrometry. The glutathiolated form of the C95A enzyme eluted earlier than the unmodified form while the disulfide-linked dimer eluted as a shoulder on the unmodified peak.

Glutathiolation of cysteine 67 and cysteine 95 was detected when treated with as low as 0.1 mM oxidized glutathione, with maximum glutathiolation reached at approximately 25 mM for C95A and 50 mM for C67A (Figure 4). At 50 mM oxidized glutathione, the glutathiolated enzyme constituted up to 45% of the original protein added (Figure 4). A comparison of the curves for the two enzymes shows that cysteine 67 is more reactive toward oxidized glutathione (Figure 4). This is likely a reflection of the surface accessibility and increased reactivity of this residue as reported previously (D'Ettorre & Levine, 1994). A time course with 20 mM oxidized glutathione demonstrated that glutathiolation of cysteine 95 (C67A) reached a maximum by 30 min with 35% being modified (Figure 5). Also, a significant amount of the enzyme forms the disulfide-linked dimer, reaching 30% within 20 min (Figure 5, inset). Formation of this dimer proceeds without glutathione present, and, in the absence of glutathione, resulted in more than 60% of the enzyme being converted to the dimer. Mono- and diglutathiolated forms of the wild-type protease were also identified by mass spectrometry following treatment with oxidized glutathione (data not shown).

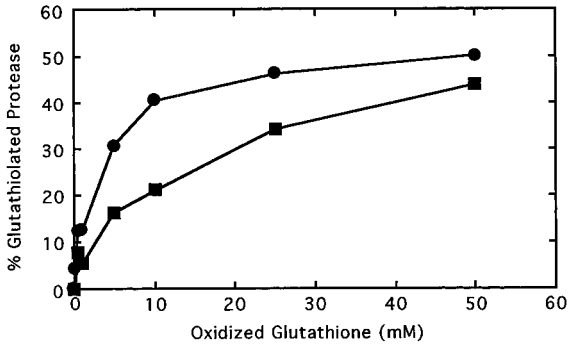

FIGURE 4: Dose response for glutathiolation of the C67A and C95A proteases. Each enzyme (25 μM for C95A and 10 μM for C67A) was treated with the indicated concentration of oxidized glutathione for 1 h and then analyzed and quantified by RP-HPLC as described under Materials and Methods. (●) C95A, enzyme glutathiolated at cysteine 67; (■) C67A, enzyme glutathiolated at cysteine 95.

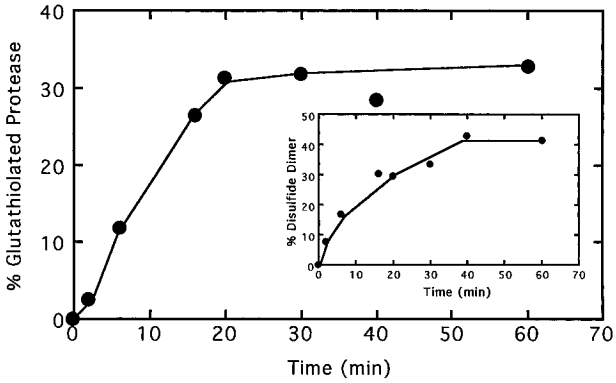

FIGURE 5: Time course for the glutathiolation of the C67A protease. The enzyme (10 μM) was treated with 20 mM oxidized glutathione at pH 7.0 for the times indicated, acidified with TFA, and then analyzed and quantified by RP-HPLC as described in the text. Inset: Time course for the formation of the disulfide-linked dimer during glutathiolation from the same experiment.

The individual proteases glutathiolated either at cysteine 67 or at cysteine 95 were collected from RP-HPLC, refolded, and assayed for protease activity. Results were compared to enzyme treated equally but without oxidized glutathione. Glutathione modification of cysteine 95 eliminated protease activity like that seen with DTNB (Figure 6). Furthermore, activity was readily restored following treatment with 10 mM DTT for 10 min (Figure 6), and this resulted in complete

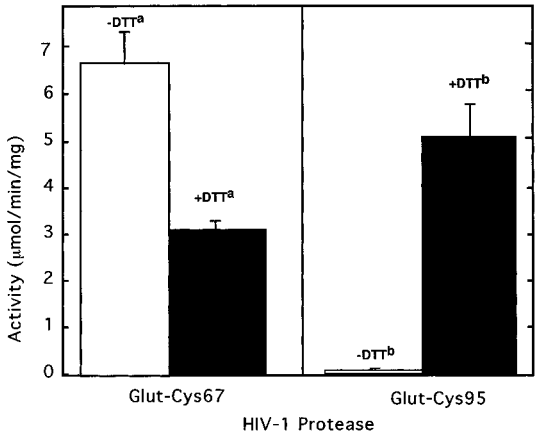

FIGURE 6: Effect of glutathiolation of cysteine 67 or cysteine 95 on protease activity and reversal with DTT. [a]The enzyme glutathiolated at cysteine 67 was refolded in the absence or presence of 10 mM DTT and then assayed (1.7 μM) for activity at pH 5.5 in a 10 min assay. [b]Enzyme (0.25−1 μM) was incubated in 225 mM sodium acetate buffer, pH 5.5, 15% glycerol, and 7.5% ethylene glycol with or without 10 mM DTT and assayed as described under Materials and Methods. Each value is the average of four separate experiments± the standard deviation.

Table 2: Effect of Glutathiolation at Cysteine 67 on Kinetic Parameters of the C95A Protease

HIV-1 protease[a]	k_{cat} (min^{-1})	K_m (mM)
control (C95A)	14 ± 4	1.6 ± 0.3
glutathiolated (C95A)	34 ± 2	2.0 ± 0.5

[a] Each enzyme was assayed for 6 min at 0.67 μM in buffer containing 150 mM sodium acetate, pH 5.5, 10% glycerol, 5% ethylene glycol, 1 M KCl. The experiment was done twice with the substrate varied from 0.5 to 5 mM. Kinetic parameters ± SD were obtained from the program Origin as described under Materials and Methods.

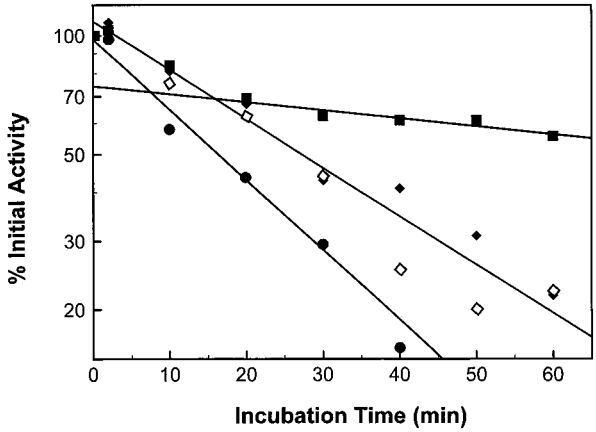

FIGURE 7: Comparison of the stability of protease activity for the C95A protease, glutathiolated C95A protease, and cysteine minus protease C67A,C95A. Each protease was incubated in buffer, and 10 μL aliquots were removed over time and assayed as described under Materials and Methods. Values are plotted on a semi-log scale as the percent activity remaining compared to the initial activity measured at 15 s. The initial activities were 3.9, 1.2, 1.6, and 3.4 μmol min^{-1} mg^{-1} for the enzyme (C95A) glutathiolated at cysteine 67, C95A without DTT, C95A with DTT, and C67A,-C95A , respectively. (■) C95A enzyme glutathiolated at cysteine 67; (◊) C95A enzyme control; (♦) C95A enzyme incubated with 5 mM DTT; (●) cysteine minus mutant protease, C67A,C95A. The regression line for the glutathiolated enzyme was calculated from the values obtained from the 20−60 min time points and extrapolated to time zero, first-order rate constant of 0.0046 min^{-1}. The regression line shown for the C95A enzyme was calculated from all the values obtained with and without DTT from 0.25−60 min time points, first order rate constant of 0.0285 min^{-1}. The regression line for C67A,C95A was calculated using the 0.25−60 min time points, first-order rate constant of 0.041 min^{-1}. Each point is the average from two separate experiments with similar results obtained in each.

removal of the bound glutathione as determined by RP-HPLC. The enzyme modified at cysteine 67 with glutathione had increased activity over the unmodified enzyme. The increase in activity was presumably due to an altered conformation adopted by the modified protein. However, an alternative possibility was that the original preparation contained high- and low-activity forms, with only the high-activity form reacting with glutathione. This glutathiolated form would be separated from the other forms by the RP-HPLC step, emerging from the subsequent refolding step as a higher activity form. This possibility might be tested by removal of the glutathione after refolding, followed by determination of specific activity. However, removal of the glutathione from the native enzyme by DTT at pH 7.0 is relatively slow (not shown), and the required longer incubation times increase autoproteolysis. Thus, the glutathiolated protein was refolded starting with 6 M guanidine with and without DTT. Refolding in the presence of DTT caused removal of over 90% of the glutathione, as determined by RP-HPLC, and a concomitant drop in enzyme activity (Figure 6). To further characterize the activation by glutathiolation at cysteine 67, a kinetic analysis was carried out. It was found that the increased activity of the glutathiolated enzyme was due to an effect on the k_{cat} (Table 2), increasing by more than 2-fold for the modified enzyme. There was little effect on K_m (Table 2).

Others have reported that incubation of the HIV-1 protease in assay buffer in the absence of substrate causes a loss in proteolytic activity, in part, due to rapid autoproteolysis but also to cysteine oxidation occurring at a slower rate (Rose et al., 1993). Thus, we examined the stability of the enzyme glutathiolated at cysteine 67 and compared it to the unmodified enzyme. Incubation of the C95A mutant in assay buffer over a 60 min period resulted in loss of activity which was first order with a rate constant of 0.0285 min^{-1} (Figure 7). The loss of activity was not significantly affected if the C95A enzyme was incubated in the presence of 5 mM DTT. In contrast, the C95A enzyme glutathiolated at cysteine 67 was substantially more stable (Figure 7). There was an initial loss in activity over the first 20 min whose rate was similar to that for the unmodified enzyme. However, enzyme activity was more stable over the next 40 min with a first-order rate constant of only 0.0046 min^{-1}. This biphasic behavior suggested the presence of two enzyme species. Reanalysis of the glutathiolated enzyme preparation by RP-HPLC indicated that 20−25% of the preparation was not modified with glutathione and this was also verified by mass spectrometry analysis. Thus, the initial loss of activity is most likely due to unmodified enzyme contained in the glutathiolated preparation as the initial rate of loss in activity matches that for the unmodified enzyme and accounts for only 25% of the total activity. To determine if the stabilization of the enzyme by glutathiolation was simply due to the loss of the free sulfhydryl, we analyzed the stability of the mutant enzyme with no cysteines (Figure 7). This change actually decreased the stability of the enzyme, yielding a first-order rate constant of 0.041 min^{-1}. Therefore, the increase in enzyme stability due to glutathiolation does not

appear to be due to protection of the reactive cysteine residue but is due to the presence of the glutathione moiety.

DISCUSSION

The two cysteines of the HIV-1 protease are highly conserved among viral isolates. Of the 22 isolates reported in the Los Alamos data base (Myers et al., 1993), all contain cysteine 95 while 19 of the 22 contain the surface-exposed residue, cysteine 67. Although neither of the two cysteine residues of the HIV-1 protease are required for catalytic activity [demonstrated here and in Loe et al. (1989), Karlstrom and Levine (1991), and Salto et al. (1994)], we have shown that mixed disulfide bond formation at either residue can have a significant effect on protease activity. Mixed disulfide bond formation at cysteine 95, either with DTNB or with glutathione, eliminates protease activity. In contrast, differential effects are observed when disulfide bond formation is made between cysteine 67 and DTNB or glutathione. While DTNB modification of cysteine 67 reduced activity, glutathiolation of this residue increased activity.

Glutathiolation at cysteine 95 presumably inhibits activity by interfering with proper dimerization of the protease. Cysteine 95 is intimately involved at the dimer interface of the protease (Wlodawer & Erickson, 1993; Manchester et al., 1994), and it has been reported that short synthetic peptides which mimic the dimer interface at this region inhibit protease activity by disrupting dimerization (Babé et al., 1992). Glutathiolation of cysteine 67 increased protease activity by increasing the k_{cat} and also stabilized the enzyme when incubated in assay buffer prior to substrate addition. The increased stability of the enzyme glutathiolated at cysteine 67 may be due to a decrease in autoproteolysis or to prevention of cysteine oxidation (Rose et al., 1993). An increase in enzyme stability may also result from a reduction in the dissociation of the protease dimer to inactive monomers known to occur with wild-type protease in the absence of substrate (Kuzmic, 1993; Kuzmic et al., 1993). The greater stability of the glutathiolated enzyme in conjunction with an increase in activity may function to regulate polyprotein processing *in vivo*.

The reactivity of cysteine 67 of the protease makes it a likely candidate for S-thiolation *in vivo*. S-Thiolation of proteins has been reported to increase in cells under oxidative stress where the levels of oxidized glutathione do not change significantly (Rokutan et al., 1991, 1994; Schuppe et al., 1992; Chai et al., 1994). S-Thiolation via oxygen radical mechanisms may be favored in HIV-1-infected cells which are reported to be in a state of oxidative stress (Ho & Douglas, 1992; Staal et al., 1992; Malorni et al., 1993), whereas reversal of protein glutathiolation is possible through enzymatic mechanisms like that carried out by the thioltransferases (Gravina & Mieyal, 1993). Interestingly, replenishing the pool of reduced glutathione in HIV-1-infected cells with antioxidants such as *N*-acetylcysteine or ascorbate has been found to suppress HIV-1 replication (Harakeh & Jariwalla, 1991; Mihm et al., 1991; Ho & Douglas, 1992; Malorni et al., 1993).

The two conserved cysteines of the HIV-1 protease could be additional targets for the development of antivirals as modification of either cysteine of the protease alters activity. Cysteine 95 is an attractive target for inhibitors as it is involved in protein−protein interactions at the dimer interface, thus tolerating few mutations without a substantial loss in protease activity (Manchester et al., 1994; Salto et al., 1994). Importantly, a haloperidol derivative has been shown to covalently modify cysteine 95, establishing the feasibility of such targeting (Salto et al., 1994).

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