Thioltransferase (Glutaredoxin) Is Detected Within HIV-1 and Can Regulate the Activity of Glutathionylated HIV-1 Protease in Vitro*

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Previous studies have suggested that the two conserved cysteines of the HIV-1 protease may be involved in regulating protease activity. Here, we examined diglutathionylated wild type protease (Cys-67-SSG, Cys-95-SSG) and the monoglutathionylated protease mutants (C67A, Cys-95-SSG and C95A, Cys-67-SSG) as potential substrates for thioltransferase (glutaredoxin). Time-dependent changes in the extent of deglutathionylation of each protein were assayed by reverse phase-high performance liquid chromatography. Glutathione alone was not an effective reductant, whereas thioltransferase displayed differential catalysis toward the Cys-95-SSG and Cys-67-SSG sites. At low thioltransferase concentrations (5 nM), deglutathionylation occurred almost exclusively at Cys-95-SSG. With substantially more thioltransferase (100 nM) Cys-67-SSG was partially deglutathionylated but only at 20% of the rate of Cys-95-SSG reduction. Treatment of the diglutathionylated protease with thioltransferase not only restored protease activity but generated an enzyme preparation that had a 3- to 5-fold greater specific activity relative to the fully reduced form. Immunoblot analysis of HIV-1MN virus with an antibody to thioltransferase detected a band co-migrating with recombinant thioltransferase that persisted following subtilisin treatment, indicating the presence of thioltransferase within HIV-1. Our results implicate thioltransferase in the regulation and/or maintenance of protease activity in HIV-1 infected cells.

Human immunodeficiency virus type 1 (HIV-1) encodes for an aspartyl protease which is required for viral maturation. This enzyme is synthesized as part of the Gag-Pol polyprotein precursor and is active as a tightly associated dimer (1). The therapeutic usefulness of potent HIV-1 protease inhibitors is based upon their ability to bind tightly to the active site of the protease dimer and block polyprotein processing, an essential step for the production of infectious virus (2). A partial decrease of protease activity in infected cells can dramatically alter the production of infectious virus (3). On the other hand, over-expression of the protease can lead to premature cell death (4–6). Studies using a highly active single-chain form of the HIV-1 protease in combination with protease inhibitors (7), as well as studies of HIV-1 containing protease mutants (8), have demonstrated the requirement for a defined range of protease activity for optimal viral production. Similar findings have been obtained for the avian sarcoma leukemia virus protease (9). Thus, retroviral polyprotein processing is a sensitive and tightly controlled process which, if disrupted, could be deleterious to viral maturation.

Recent studies have indicated that the two cysteines of the HIV-1 protease may be involved in redox regulation of protease activity (10). The two cysteine residues are highly conserved among HIV-1 isolates from patients. Of 31 different HIV-1 isolates spanning 6 different major subtypes, all contain cysteine 95, whereas 29 contain cysteine 67 (11). By contrast, the HIV-2 protease does not contain conserved cysteine residues, suggesting that the cysteine residues of the HIV-1 protease are not absolutely required for retroviral polyprotein processing but rather may offer a selective advantage for HIV-1 in vivo. Under native conditions, both cysteines of the HIV-1 protease are unusually susceptible to oxidation (12). Modification of either cysteine residue of the HIV-1 protease with sulfhydryl modifying reagents, such as 5,5'-dithiobis(2-nitrobenzoic acid), leads to a decrease or loss in protease activity (10, 12–15). Additional studies, utilizing protease mutants produced by site-directed mutagenesis, demonstrated that mixed disulfides between the cysteine residues and glutathione (glutathionylation) have dramatic effects on protease activity. Glutathionylation of cysteine 67, a solvent exposed residue, increased activity several fold and also stabilized the activity in vitro. However, glutathionylation of cysteine 95, located at the dimer interface, abolished protease activity (10). In vivo, cysteine modification of proteins with glutathione increases in cells under conditions of oxidative stress (16–18), which would make the cysteines of the HIV-1 protease likely candidates for glutathionylation in HIV-1-infected cells.

The reversible nature of glutathionylation and its effects on HIV-1 protease activity led us to investigate the possible involvement of human thioltransferase in regulating the redox status of these cysteine residues. Thioltransferase (EC 1.8.4.2) (also known as glutaredoxin) belongs to the class of enzymes termed thiol-disulfide oxidoreductases, which include thioredoxin and protein disulfide isomerase (19–21). The prevalence of S-glutathionylation of proteins under conditions of oxidative stress has focused attention on the role of thioltransferase in maintaining the activities of important cellular proteins that are altered by glutathionylation (20–24). In this light, we hypothesized that thioltransferase may play a similar role for the HIV-1 protease under conditions favoring glutathionylation.

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§ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; GSH, glutathione; RP-HPLC, reverse phase-high performance liquid chromatography; DTT, dithiothreitol; WT, wild type; MES, 4-morpholineethanesulfonic acid; TCEP, tris(2-carboxyethyl)phosphine HCl.
during viral replication. In the current study, we discovered that thioltransferase preferentially deglutathionylates cysteine 95 of the HIV-1 protease leading to modulation of protease activity. In addition, we report, for the first time, the detection of this mammalian protein within HIV-1 virions.

**EXPERIMENTAL PROCEDURES**

**HIV-1 Proteases and Thioltransferase**—Recombinant HIV-1 protease (strain HXB2) and two HIV-1 protease mutants (C67A and C95A) were expressed and purified from *Escherichia coli* as described previously (10). Protease activity was assayed using a 9 amino acid peptide spanning the p17/24 junction in the HIV-1 Gag protein as the substrate, as described previously (10). Assays for protease activity were carried out in 100–200 mM sodium phosphate buffer, pH 6.2, containing 1 mM EDTA, 10% glycerol, 5% ethylene glycol for 1–5 min, as indicated, with a final substrate concentration of 2 mM. Recombinant human thioltransferase (57 units/mg) was expressed in *E. coli*, purified and assayed as described previously (25), and stored at −70 °C in 20% glycerol.

*In Vitro Glutathionylation of the HIV-1 Proteases*—The HIV-1 wild type protease and mutant proteases (C67A and C95A) were refolded and stored in 20 mM HCl at pH 1.6 at 0.2–1.5 mg ml⁻¹ and stored in 20 mM HCl at pH 1.6 at 0.2–1.5 mg ml⁻¹ as described previously (10). To glutathionylate the cysteines, the proteases were incubated at 37 °C for 2 h in a buffer containing 250 mM Tris-HCl at pH 7.8, 6.0 g guanidine, 1 mM disodium EDTA and 40 mM glutathione disulfide. The reaction was stopped by acidification to pH < 2.0 with trifluoroacetic acid. More than 80% of both cysteines were glutathionylated using this procedure. The glutathionylated proteases were purified (>95%) from residual unmodified protease by RP-HPLC by exploiting the change in retention time imparted by each glutathionyl moiety. Each of the 3 glutathionylated proteases were separated on a Vydac C18 column using a gradient method with solvent A, consisting of deionized water containing 0.05% trifluoroacetic acid, and solvent B, consisting of acetonitrile containing 0.05% trifluoroacetic acid. The column was eluted at 1 ml min⁻¹ and the percent of solvent B was increased linearly from 5 to 35% over 5 min followed by an increase to 57.5% solvent B over the next 15 min; solvent B was then increased to 95% over 2 min and then ramped back to the initial starting conditions over the final 5 min of the gradient program. Protease elution was monitored at both 205 and 276 nm. The location of the peaks from RP-HPLC corresponding to the glutathionylated forms of the C67A and C95A mutant proteases had been identified previously by electrospray mass spectrometry (10). Following purification by RP-HPLC, all proteases were refolded as described previously (14), except that DTT was omitted to prevent reduction of the glutathionyl disulfides. Protein concentrations were calculated using the molar absorptivity (ε280 nm = 12,800 M⁻¹ cm⁻¹).

**HITCL Analysis of Glutathionylated and Unmodified HIV-1 Proteases**—To test the proteases as substrates for human thioltransferase, the glutathionylated forms of the protease (at 1–4 μM) were incubated in 200 mM sodium phosphate buffer, pH 6.2, containing 1 mM EDTA, 10% glycerol, 5% ethylene glycol, 60 μg ml⁻¹ of bovine serum albumin and 0.5 mM reduced glutathione (unless otherwise indicated). Thioltransferase was added, and the solution was incubated at 37 °C for various times, and where indicated in text aliquots were assayed for protease activity. The reaction was stopped by acidification (pH < 2) with trifluoroacetic acid. The samples were then analyzed by RP-HPLC, and the areas of absorbance peaks (205 nm) corresponding to the glutathionylated and deglutathionylated proteases were used to determine the extent of deglutathionylation at each cysteine residue. Immuno blot Analysis of Viral Preparations—HIV-1 gag samples were obtained from the Clone 4 cell line (26) and digested with subtilisin as described previously (27). The treated viral preparation was then repurified by sucrose banding. Viral samples were treated with 50 mM DTT and 20 mM TCEP (Calbiochem) to maintain the cysteine residues of thioltransferase in their reduced form. Samples were electrophoresed on a 10% Bis-Tris polyacrylamide gel with MES running buffer using the NuPage system from Novex (San Diego, CA). Proteins were electroblotted onto nitrocellulose, and thioltransferase was detected using an anti-glutaredoxin antibody obtained from American Diagnostica, Inc. (Greenwich, CT). Monoclonal antibodies to gp120 were obtained from Intracel (Cambridge, MA). For competition experiments, 20 μg of glutaredoxin antibody were preincubated at room temperature in the presence or absence of 6 μg of purified thioltransferase for 2 h at a dilution of 1:50, followed by centrifugation at 10,000 rpm for 10 min to eliminate thioltransferase-bound antibodies.

**RESULTS**

**HPLC Analysis of Glutathionylated and Unmodified HIV-1 Proteases**—The wild type HIV-1 protease and the C67A and C95A mutants were each glutathionylated at their respective cysteine residues, purified by RP-HPLC, and refolded as described previously (14) for use as substrates for human thioltransferase. The glutathionylated form of each protease monomer had a distinct retention time on the C8 column. The difference in retention times possibly reflects varying degrees of interaction with the C8 column of the surface exposed area around cysteine 67 of the protease versus that for the dimer interface region around cysteine 95.

**FIG. 1.** RP-HPLC analysis of wild type and mutant HIV-1 proteases. Top, wild type HIV-1 protease (dotted line) and wild type HIV-1 protease glutathionylated at both cysteine 67 and cysteine 95 (solid line); center, C95A protease (dotted line) and C95A protease glutathionylated at cysteine 67 (solid line); bottom, C67A protease (dotted line) and C67A protease glutathionylated at cysteine 95 (solid line). Each tracing was obtained with 1–10 μg of purified protease and all were normalized to the same absorbance at 205 nm to aid comparison. This analysis reveals that each protease has a distinct retention time on the C8 column. The difference in retention times possibly reflects varying degrees of interaction with the C8 column of the surface exposed area around cysteine 67 of the protease versus that for the dimer interface region around cysteine 95.
Thioltransferase Preferentially Deglutathionylates Cysteine 95 of the HIV-1 Protease—Thioltransferase readily catalyzed the GSH-dependent deglutathionylation of the C67A,Cys-95-SSG (glutathionylated at cysteine 95) protease as determined by RP-HPLC analysis (Fig. 2). Further experiments demonstrated the requirement for thioltransferase catalysis, because glutathione alone (0.5 mM) showed no measurable reduction of cysteine 95 during a 10 min time course, whereas nearly 30% was deglutathionylated when 5 mM thioltransferase was added with GSH (Fig. 2). As reported previously, the C95S-SSG protease is inactivated by the glutathionyl moiety (10). However, treatment of the inactivated protease with thioltransferase resulted in the restoration of protease activity in a time-dependent manner reflecting the rate of deglutathionylation (Fig. 2). This observation is also consistent with previous results showing that reduction of C67A,Cys-95-SSG with 10 mM DTT could restore protease activity.

In contrast to the results with the C67A,Cys-95-SSG protease, the C95A,Cys-67-SSG (glutathionylated at position 67) protease was a poor substrate for thioltransferase. Treatment with 5 mM thioltransferase did not lead to measurable deglutathionylation of cysteine 67 over a 10 min assay (Fig. 2), nor did it significantly alter protease activity (Fig. 2). C67A,Cys-67-SSG, however, was not completely resistant to thioltransferase treatment. Exposure of the C67A,Cys-67-SSG protease to a much higher concentration of thioltransferase (100 nM) for 5 min deglutathionylated up to 20% of the C67-SSG protease compared with 60% for the C67A, Cys-95-SSG protease. These studies indicated that thioltransferase preferentially catalyzes the deglutathionylation of cysteine 95 of the HIV-1 protease.

The wild type protease, WT-(SSG)2 (glutathionylated at both cysteine 67 and cysteine 95) (so that the dimeric protease is tetraglutathionylated), was then tested as a substrate for thioltransferase. Based on the results with the mutant proteases, one would predict that the WT-(SSG)2 protease would be selectively deglutathionylated at cysteine 95 in the presence of thioltransferase. Treatment of the WT-(SSG)2 protease with 20 mM thioltransferase and 0.5 mM GSH for 5 min resulted in a decrease in the area for the WT-(SSG)2 peak and the generation of an additional peak in the RP-HPLC chromatogram along with two minor peaks eluting later (Fig. 3, top panel). The new peak eluted approximately 0.5 min later than that assigned to the WT-(SSG)2 protease, and the mass obtained by electrospray mass spectrometry was consistent with a monogluthationylated form of the protease monomer (expected 11,083 Da; obtained 11,084 Da). The retention time for this new peak corresponded closely to the retention time for the C95A,Cys-67-SSG protease mutant (retention time of 15.6 min compared with 15.8 min for the mutant, see Fig. 1), suggesting that the new peak represented the conversion of part of the WT-(SSG)2 protease to the WT-Cys-95-SH,Cys-67-SSG protease generated by deglutathionylation at position 95 but not at position 67. If so, we would hypothesize that thioltransferase treatment of the WT-(SSG)2 protease would restore protease activity.

Thioltransferase Activates Protease Activity of the WT-(SSG)2 Protease—To determine if thioltransferase restored protease activity for the WT-(SSG)2 protease, we measured protease activity following treatment with 5 nM thioltransferase for the C67A protease (broken line) and for the C95A protease (glutathionylated at Cys-67) (solid line).

FIG. 2. Deglutathionylation of HIV-1 protease mutants with thioltransferase and its effect on protease activity. C67A HIV-1 protease mutant (glutathionylated at Cys-95) or C95A HIV-1 protease mutant (glutathionylated at Cys-67) was treated with 5 nM thioltransferase. Aliquots were removed at 0, 5, and 10 min and acidified with trifluoroacetic acid or analyzed for protease activity for 1 min as described under “Experimental Procedures.” The extent of deglutathionylation at each cysteine residue was determined by RP-HPLC by obtaining the areas for the glutathionylated and deglutathionylated peaks. Each value is the average of two experiments with similar results. ( ), extent of deglutathionylation following treatment with 5 nM thioltransferase for the C67A protease (glutathionylated at Cys-95) (broken line) and for the C96A protease (glutathionylated at Cys-67) (solid line). ( ), protease activity following treatment with 5 nM thioltransferase for the C67A protease (broken line) and for the C95A protease (glutathionylated at Cys-67) (solid line).

FIG. 3. Deglutathionylation and activation of protease activity for the WT-(SSG)2 protease following treatment with thioltransferase. Top, RP-HPLC tracing for the WT-(SSG)2 protease incubated in buffer for 5 min in the absence of thioltransferase (dotted line), and RP-HPLC tracing for the WT-(SSG)2 protease (5 μM) incubated in buffer for 5 min containing 20 nM thioltransferase (solid line). The peaks for the diglutathionylated (WT-(SSG)2) protease, the monogluthationylated protease (WT-SSG), and the fully reduced wild type protease (WT) are labeled in the figure. Aliquots of the untreated and treated WT-(SSG)2 protease samples were tested for protease activity (top, inset) as described under “Experimental Procedures.” Bottom, RP-HPLC tracing for the WT-(SSG)2 (3 μM) protease following incubation in buffer for 10 min in the presence of 100 nM thioltransferase (solid line). An aliquot of the thioltransferase-treated sample was analyzed for protease activity (bottom, inset) as described under “Experimental Procedures.” TTase, thioltransferase.
activity following thioltransferase treatment of the inactive WT-(SSG)₂ protease, expecting that selective deglutathionylation at cysteine 95 would restore protease activity. As predicted, thioltransferase treatment of the WT-(SSG)₂ protease did reactivate the HIV-1 protease (Fig. 3, top panel, inset). The specific activity was 2.3 μmol/min/mg based on total protein (Fig. 3, top panel, inset) but it was 8.1 μmol/min/mg when calculated based on the amount of protein associated with that of the new peak generated by thioltransferase treatment (Table I). The change in protease activity for the WT-(SSG)₂ protease resulting from thioltransferase treatment correlated closely with the extent of deglutathionylation as assessed by RP-HPLC (Table I). Calculating specific activity based on the percentage of protease deglutathionylated to active forms by thioltransferase (which includes those forms deglutathionylated at cysteine 95 as determined by RP-HPLC analysis) yielded a specific activity approximately 3- to 5-fold greater than that expected for the TCEP-treated enzyme (Table I). These data are consistent with our previous studies, which showed that glutathionylation of C95A protease at cysteine 67 significantly increased protease activity as compared with wild type (10). Thus, we conclude that selective glutathionylation of the wild type enzyme at cysteine 67 may result in a form of the enzyme with significantly greater protease activity than unmodified wild type protease.

Deglutathionylation of the WT-(SSG)₂ protease was also studied as a function of GSH concentration. In the absence of GSH, no deglutathionylation was observed (Fig. 4). Measurable deglutathionylation of the WT-(SSG)₂ protease by GSH alone was not detected until more than 1 mM GSH was present, whereas the thioltransferase-catalyzed reaction was already half-maximal at less than 0.1 mM GSH (Fig. 4). Based on these data, thioltransferase (20 nM), in the presence of glutathione, was more than 50,000 times more potent than GSH alone (>1 mM) at deglutathionylating cysteine 95.

### Table I

| Treatment of WT-(SSG)₂ protease | Protease activity | % Protease deglutathionylated at Cys-95 | Specific Activity β | µmol/min/mg |
|--------------------------------|------------------|----------------------------------------|--------------------|-------------|
| None                           | 3                | <4                                     | NA                 |             |
| Thioltransferase               |                  |                                        |                    |             |
| 5 nM                           | 1166             | 10.0                                   | 11.8               |             |
| 10 nM                          | 1756             | 14.7                                   | 12.1               |             |
| 20 nM                          | 2242             | 26.8                                   | 8.5                |             |
| TCEP (20 mM)                   | 2422             | 96.2                                   | 2.6                |             |

a Specific activity was calculated based on the percent of the total enzyme that was converted to active forms as assessed by RP-HPLC (the monogluthathionylated form of the protease and the wild type unmodified form). In the case of TCEP treatment, >90% of the total enzyme was converted to the fully reduced form, and the specific activity obtained can be used to compare with that of the preparations treated with thioltransferase.

b NA, not applicable; trace levels of deglutathionylated protease present cannot be accurately assessed by RP-HPLC. The data correspond to one representative experiment; other experiments gave similar results.

GSH alone at concentrations as high as 10 mM did not result in measurable deglutathionylation of the cysteine 67 residue of the WT-(SSG)₂ protease. In contrast, treatment with 10 mM DTT resulted in partial deglutathionylation at both cysteine 67 and cysteine 95 as shown by the presence of four peaks in the RP-HPLC chromatogram representing the four different redox forms of the wild type protease monomer (not shown). The same four peaks were obtained when the WT-(SSG)₂ protease was treated with 100 nM thioltransferase (Fig. 3, bottom panel). In this case, the protease specific activity was 5.2 μmol/min/mg based on total protein but is 8.3 μmol/min/mg when calculated based on the amount of active forms of the enzyme present in the mixture. This value is still significantly higher than that for the fully reduced wild type protease and may indicate progressive deglutathionylation of the tetraglutathionylated wild type protease dimer. This could generate partially glutathionylated heterodimers as intermediates that are more active than the fully reduced dimeric protease. These studies show that the enzymatic deglutathionylation by thioltransferase is much more efficient than chemical deglutathionylation by DTT on a molar basis (100 nM thioltransferase versus 10 mM DTT, i.e. 100,000 times), and the thioltransferase-catalyzed reaction displayed a clear preference for cysteine 95, which is consistent with the data for the individual mutant proteases.

**Thioltransferase Is Detected Within HIV-1 Virions**—The activation of protease activity from the selective deglutathionylation of cysteine 95 of the HIV-1 protease by human thioltransferase suggested a possible role for this enzyme in HIV-1 replication. It has been shown that vaccinia virus and T4 phage both encode and package their own glutaredoxins, which are functional as thioltransferases (29–31). Thus, it seemed possible that HIV-1 might package human thioltransferase in a similar manner. To test this hypothesis, we utilized viral preparations from an H9 cell clone (26) that produces virions in the absence of significant levels of cellular microvesicles that would contaminate the preparation with cellular proteins as indicated by others (32). Such preparations had previously been employed to identify the presence of cyclophilin A in HIV-1 (27). When such a virion preparation is treated with subtilisin, proteins found on the outside of HIV-1 virions are digested,
Thioltransferase Regulates Glutathionylated HIV-1 Protease

Cysteine residues in proteins serve a number of important roles, including catalysis (33), protein folding (34), and DNA binding (35). Cysteines can also play an important role in the regulation of enzyme activity like that described for citrate synthase and adenovirus protease (33, 36–38). The highly conserved nature of the two cysteine residues of the HIV-1 protease, which are neither directly involved in the catalytic mechanism nor involved in intramolecular disulfide bond formation, suggests a role for these residues in the regulation or maintenance of protease activity.

We found that thioltransferase was detectable in virion preparations following subtilisin digestion, providing evidence that this enzyme is present within virions. Other host proteins have been identified previously within HIV-1 virions including cyclophilin A, which is essential for viral infectivity (11, 27, 51–53). Immunoblot analysis indicated very low levels of thioltransferase, suggesting passive acquisition of cellular thioltransferase by the virus. It would be of interest to determine if thioltransferase within HIV-1 virions offers an advantage in regards to viral infectivity. Interestingly, vaccinia virus and T4 bacteriophage each encode two different functional glutaredoxins that have thioltransferase activity (29–31, 54). Although the role of the glutaredoxins (thioltransferases) in the viral life cycle is uncertain, their presence in the virus supports the argument that thioltransferase may play a fundamental role in optimizing HIV-1 replication.

Several roles for thioltransferase in viral replication are conceivable, including homeostatic or regulatory control of the
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Glutathionylation status of sulfhydryl groups on viral proteins, including metabolic enzymes and transcription factors (20). Thioltransferase could also affect the glutathione-dependent folding of viral proteins containing cysteine residues, including the HIV-1 protease (34). Finally, thioltransferase could protect important viral proteins that are oxidatively damaged during oxidative stress. In particular, inactivation of the protease through disulfide bond formation may explain the inability to completely restore polyprotein processing in immature virions once they have been released from cells (55). Our studies suggest that the cysteine residues of the HIV-1 protease coupled through disulfide bond formation may explain the inability to oxidatively damage during the HIV-1 protease (34). Finally, thioltransferase could protect the cysteine residues of the HIV-1 protease coupled with thioltransferase and GSH may optimize HIV-1 protease activity particularly under conditions of oxidative stress.

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