HIV-2 protease is inactivated after oxidation at the dimer interface and activity can be partly restored with methionine sulfoxide reductase

David A. DAVIS*, Fonda M. NEWCOMB*, Jakob MOSKOVITZ†, Paul T. WINGFIELD‡, Stephen J. STAHL‡, Joshua KAUFMAN‡, Henry M. FALES§, Rodney L. LEVINE† and Robert YARCHOAN*

*HIV and AIDS Malignancy Branch, National Cancer Institute, Building 10, Room 12N226, 9000 Rockville Pike, National Institutes of Health, Bethesda, MD 20892-1906, U.S.A., †Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, Building 3, Room 106, 9000 Rockville Pike, National Institutes of Health, Bethesda, MD 20892, U.S.A., ‡Protein Expression Laboratory, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Building 6B, Room 1B130, 9000 Rockville Pike, National Institutes of Health, Bethesda, MD 20892, U.S.A., and §Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, Building 10, Room 7N318, National Institutes of Health, Bethesda, MD 20892-1676, U.S.A.

Human immunodeficiency viruses encode a homodimeric protease that is essential for the production of infectious virus. Previous studies have shown that HIV-1 protease is susceptible to oxidative inactivation at the dimer interface at Cys-95, a process that can be reversed both chemically and enzymically. Here we demonstrate a related yet distinct mechanism of reversible inactivation of the HIV-2 protease. Exposure of the HIV-2 protease to H_2O_2 resulted in conversion of the two methionine residues (Met-76 and Met-95) to methionine sulfoxide as determined by amino acid analysis and mass spectrometry. This oxidation completely inactivated protease activity. However, the activity could be restored (up to 40%) after exposure of the oxidized protease to methionine sulfoxide reductase. This treatment resulted in the reduction of methionine sulfoxide 95 but not methionine sulfoxide 76 to methionine, as

determined by peptide mapping/mass spectrometry. We also found that exposure of immature HIV-2 particles to H_2O_2 led to the inhibition of polyprotein processing in maturing virus particles comparable to that demonstrated for HIV-1 particles. Thus oxidative inactivation of the HIV protease *in vitro* and in maturing viral particles is not restricted to the type 1 proteases. These studies indicate that two distinct retroviral proteases are susceptible to inactivation after a very minor modification at residue 95 of the dimer interface and suggest that the dimer interface might be a viable target for the development of novel protease inhibitors.

Key words: polyprotein processing, redox regulation, retroviruses, thioredoxin, viral maturation.

INTRODUCTION

Many proteins are susceptible to reversible inactivation after oxidation of critical cysteine and/or methionine residues. One modification of cysteine residues that takes place in cells is reversible disulphide bond formation with the cellular thiol, glutathione (termed glutathiolation) [1–3]. A number of studies indicate that the glutathiolation of cellular proteins (glyceraldehyde-3-phosphate dehydrogenase, BSA, haemoglobin, nuclear factor 1) can be reversed *in vitro* in the presence of the cellular enzyme thioltransferase (glutaredoxin) [4–9]. It has been suggested that this enzyme might perform the repair of oxidative damage that occurs to proteins *in vivo* [10,11]. In contrast, methionine residues in proteins are susceptible to oxidation through the oxidation of the thioether moiety, leading to the formation of methionine sulfoxide [12,13]. The reversal of methionine oxidation *in vitro* can take place for certain proteins (α -1-proteinase inhibitor, ribosomal protein L12 and voltage-dependent K^+ channel) in the presence of the cellular enzyme methionine sulfoxide reductase (MsrA) [13–15]. Studies involving an MsrA mutant in yeast cells indicate that this enzyme functions to restore oxidative damage *in vivo* after oxidative stress [16,17]. MsrA has also been reported to be important in host–pathogen interactions, for example in maintaining the

function of adhesion proteins required for the pathogenicity of three different bacteria [18].

In our previous studies we demonstrated that the HIV-1 protease could be reversibly inactivated after the glutathiolation of Cys-95 located at the dimer interface. Furthermore, thioltransferase, a cellular enzyme, was found to be capable of preferentially removing glutathione from Cys-95 and restoring enzyme activity. Because thioltransferase was also detected within HIV-1 particles released from T-cells, we suggested that thioltransferase might function to maintain or regulate protease activity in HIV-1-infected cells under oxidative stress. However, the fact that the closely related HIV-2 protease was not found to contain cysteine residues suggested that this mechanism might be specific for HIV-1 and thus brought into question the significance of this potential oxidative inactivation. However, further inspection of the HIV-2 protease sequences revealed that this enzyme contains two completely conserved methionine residues, one of which (Met-95) is located at the same position in the dimer interface as Cys-95 in the HIV-1 protease. Although methionine is, in general, not considered a conservative substitute for cysteine the possibility remained that it functioned to inhibit the HIV-2 protease after oxidation. Therefore in this study we examined the possibility that the HIV-2 protease might be reversibly inhibited after the oxidation of Met-95 located at the dimer interface.

Abbreviations used: DTT, dithiothreitol; ES–MS, electrospray MS; MsrA, methionine sulfoxide reductase; RP–HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail dadavis@helix.nih.gov).

EXPERIMENTAL

Proteins

Recombinant, affinity purified, HIV-2_{ROD} protease expressed in *Escherichia coli* was purchased from Bachem (Torrance, CA, U.S.A.). Yeast MsrA EC 1.8.4.6 was prepared as described previously [17]. The HIV-1 proteases used in this study were wild-type (sequence from strain HXB2) and the following mutants: C67A C95A and C67A C95M. C67A C95M protease, isolated similarly to that described previously [19], was further purified by reverse-phase HPLC (RP-HPLC) because it contained larger amounts of uncleaved precursors. Trypsin was purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of oxidized proteases

HIV-2 protease and C67A C95M protease were treated with H₂O₂ at a final concentration of 200 mM in 20 mM HCl, pH 1.65, for 10 min at 37 °C to prepare the oxidized forms of the proteases. When proteins are incubated under acidic conditions, the methionine residues are specifically oxidized by H₂O₂, leaving the other amino acids intact [12]. The modified enzymes were then dialysed three times for 1 h against a 1000-fold excess of storage solution (20 mM HCl, pH 1.65) to remove the remaining H₂O₂. The protein concentrations were determined spectrophotometrically (ϵ_{280} 12 300 M⁻¹·cm⁻¹ for HIV-1 protease; ϵ_{280} 9150 M⁻¹·cm⁻¹ for HIV-2 protease). Protease samples not exposed to H₂O₂ were otherwise treated identically and used as the unoxidized controls. All samples were stored in aliquots at -70 °C. RP-HPLC was used to separate the oxidized forms of the proteases and in conjunction with MS to determine the extent of oxidation of the proteases. The C67A C95A and C67A C95M HIV-1 proteases were analysed by RP-HPLC as described previously [19]. The wild-type HIV-2 protease (3 µg) before and after oxidation with H₂O₂ was separated on a Vydac C₁₈ (2.1-mm internal diam. × 500 mm, 5 µm particle size) column by a gradient method with deionized water containing 0.05% trifluoroacetic acid (solvent A) and acetonitrile containing 0.05% (v/v) trifluoroacetic acid (solvent B). The column was eluted at 0.300 ml/min and the percentage of solvent B was increased linearly from 5% to 35% over 5 min followed by an increase to 65% solvent B over the following 15 min. The elution of the protease was monitored at 205 and 276 nm. The peaks were collected and analysed by MS as described below to determine the extent of oxidation. Oxidation of the methionine residues of HIV-2 protease was verified by amino acid analysis on 1 µg of protein by using the CNBr method described previously [20]. MS was performed as described previously with a TSQ700 spectrophotometer (Finnigan, Sunnyvale, CA, U.S.A.) [19] and used to confirm the oxidation of the proteases.

Enzymic assays

Determinations of HIV-1 and HIV-2 protease activity were performed as described previously [19]. Proteases at final dimeric concentrations ranging from 0.25 to 1.5 µM were incubated at 37 °C in a protease assay buffer containing 150 mM sodium acetate, pH 5.7, 10% (v/v) glycerol, 5% (v/v) ethylene glycol, 1 mM EDTA, 100 µg/ml acetylated BSA and 3 mM substrate. Measurements of protease activity were taken after incubation for 5 and 10 min to verify linearity of the assays. The assay was stopped with an aliquot of concentrated (10%, v/v) trifluoroacetic acid and the products were determined by RP-HPLC as described previously [19]. Protease activity is reported as the average specific activity from the two time points; values of

the specific activity between the two time points were always within 10% of each other.

To attempt to reverse the oxidative modifications of the proteases, samples were treated with MsrA in conjunction with dithiothreitol (DTT) or the thioredoxin/thioredoxin reductase system [21]. The protease samples (4 µM final concentration) were incubated in 150 mM sodium acetate buffer, pH 6.0, containing 500 µM EDTA and 40 mM DTT. MsrA was added at the concentrations indicated in the Results section and the samples were incubated for 10 min at 37 °C. Aliquots were then taken for analysis of protease activity as described above or were used in peptide mapping experiments described below.

Proteolytic digests of HIV-1 and HIV-2 proteases and RP-HPLC/MS analysis

RP-HPLC/MS analyses of peptide digests of the proteins were performed to determine the extent of reversal of methionine oxidation by MsrA. After exposure of the unoxidized or oxidized HIV-2 protease to different concentrations of MsrA, samples of the proteases (5–10 µg) were brought to pH 8.0 with 1 M Tris/HCl. Acetonitrile was then added to a final concentration of 20% and the sample was then treated overnight with either trypsin (0.25 µg) or Lys C (0.25 µg) at 37 °C. The samples were dried by vacuum centrifugation and suspended in 6 M guanidine, pH 2.0, containing trifluoroacetic acid; the peptides were analysed by RP-HPLC/MS. Electrospray MS (ES-MS) was performed with a Hewlett-Packard model G1946A instrument interfaced to a model 1100 HPLC system equipped with a Vydac 218TP narrow-bore C₁₈ column. The effluent from the column (200 µl/min) was mixed in a tee junction with acetic acid/acetonitrile (50:50), pumped by another 1100 series pump (100 µl/min), and the mixture was introduced into the mass spectrometer [22].

Preparation of immature viral particles from infected H9 cells and viral polyprotein processing experiments

Immature HIV-1_{LAI} or HIV-2_{EH0} viral particles were prepared from chronically infected H9 cells treated with the protease inhibitor KNI-272 as described [23]. For polyprotein processing experiments, 1 ml aliquots of the immature HIV-1 or HIV-2 were thawed at room temperature and then centrifuged at 100 000 g for 30 min. To decrease the concentration of protease inhibitor substantially, the supernatant was removed by aspiration and the viral pellets were each resuspended in 1 ml of fresh medium maintained at 4 °C, then centrifuged as before. This washing step was then repeated once and each viral pellet was resuspended in 25 µl of conditioned medium [23]. Polyprotein processing of immature HIV-1 and HIV-2 particles was initiated by incubating 10 µl aliquots of the immature viral particles in conditioned medium at 37 °C for 6 h; this was done in the absence or presence of increasing concentrations of H₂O₂. After 6 h, polyprotein processing was terminated with an equal volume of 2×SDS sample buffer (Novex, San Diego, CA, U.S.A.) containing 100 mM DTT. These samples were analysed for polyprotein processing by Western blot analysis of the mature viral capsid protein p24 for HIV-1 and p26 for HIV-2.

Western blot analysis of viral proteins

Samples for Western blot analysis were subjected to electrophoresis on a Bis-Tris/10% (w/v) polyacrylamide gel with Mes running buffer by using the NuPage system from Novex (San Diego, CA, U.S.A.). Proteins were electroblotted to nitrocellulose

and the mature capsid protein p24 for HIV-1 and p26 for HIV-2 were detected with the use of a mouse monoclonal anti-p24 antibody from ABI (Columbia, MD, U.S.A.) [23] or a mouse monoclonal anti-p26 antibody obtained from Intracel (Cambridge, MA, U.S.A.). Densitometry of the bands was done as described [23].

RESULTS

Exposure of HIV-2 protease to H_2O_2 results in enzyme inactivation through oxidation of residue 95 at the dimer interface

The 99-residue HIV-2 protease does not contain the two highly conserved cysteine residues located at positions 67 and 95 in HIV-1 protease (Figure 1, lower panel). However, analysis of HIV-2 protease sequences in the protein database [24] revealed that the HIV-2 protease sequences examined so far contain two completely conserved methionine residues (residues 76 and 95). In particular, Met-95, like Cys-95 of HIV-1 protease, is located at the dimer interface (Figure 1, upper panels). Substitution of methionine for a cysteine residue in a protein is usually considered a non-conservative amino acid change. However, cysteine and methionine each contain a sulphur that can be susceptible to reversible oxidative modification [13]. To examine whether oxidative modification of the methionine residues of HIV-2 protease with H_2O_2 would inhibit enzyme activity, we first exposed the protease for a brief period (10 min) to high (200 mM) concentrations of H_2O_2 under acidic conditions. This method is used to ensure preferential modification of the methionine residues [12]. ES-MS confirmed that the mass of HIV-2 protease increased by 32 atomic mass units (amu) after exposure to H_2O_2 [untreated enzyme, 10720 amu obtained (10718.6 expected) compared with H_2O_2 -treated enzyme, 10752 amu obtained (10750.6 expected)], which was consistent with the addition of two oxygens to the protein. Amino acid analysis performed as

Figure 1 Molecular models of HIV-1 and HIV-2 protease and sequence comparison of HIV-1 and HIV-2 proteases

Upper panel: left, ribbon drawing for the HIV-1 protease, showing the location of the two conserved cysteine residues at positions 67 and 95 (as ball and stick in black); right, ribbon drawing for the HIV-2 protease showing the location of the two conserved methionine residues at positions 76 and 95 (as ball and stick in black). The two monomers are denoted by a and b. Co-ordinates are from the Protein Data Bank, accession nos. 1JLD for HIV-2 protease and 1ODW for HIV-1 protease. The molecular structures were represented by RASMOL [30]. Lower panel: alignment of the major consensus sequence for HIV-1 protease (consensus B) with HIV-2_{ROD} sequence (consensus A) [24]. HIV-2 protease has 48% sequence identity and 72% sequence similarity to HIV-1 protease. The cysteine and methionine residues are shown in bold.

Figure 2 RP-HPLC profile and protease activity after exposure of HIV-2 protease to H_2O_2

Untreated (solid line) and H_2O_2 -treated (dotted line) wild-type HIV-2 protease were analysed by RP-HPLC and for protease activity as described in the Materials and methods section. ES-MS analysis indicated an increase of 32 amu (MU) for the treated HIV-2 protease, which was consistent with the mono-oxidation of both methionine residues. Inset: the untreated and treated HIV-2 proteases were assayed for 5 and 10 min in the presence of peptide substrate and the average specific activity was determined as described in the Materials and methods section.

Figure 3 Restoration of HIV-2 protease activity after exposure of the inactive oxidized protease to MsrA

Oxidized HIV-2 protease was treated for 10 min at 37 °C in protease assay buffer with the indicated concentrations of MsrA. Peptide substrate was then added and protease activity was determined. The relative protease activity was calculated on the basis of the activity of the unoxidized form of the protease, which was otherwise treated and assayed under identical conditions in the absence of MsrA. The activity for the unoxidized HIV-2 protease was 2.2 $\mu\text{M/min per mg}$. A dose-response experiment was performed on three different occasions with similar results; a representative experiment is shown.

described to determine the extent of methionine oxidation [20] confirmed that both methionine residues in the protein were preferentially oxidized after exposure to H_2O_2 (results not shown). This oxidation resulted in a shift to an earlier retention time for the protein on RP-HPLC and completely eliminated protease activity, which was consistent with oxidation of the protein (Figure 2). RP-HPLC/MS analysis of tryptic digests of the oxidized HIV-2 protease confirmed that the methionine-containing peptides each increased by 16 amu as a result of exposure to H_2O_2 (results not shown). Taken together, these studies indicate that oxidation of the Met-76 and Met-95 in HIV-2 protease is sufficient to inhibit protease activity.

Table 1 Restoration of HIV-2 protease activity after exposure of oxidized HIV-2 protease to MsrA

Oxidized HIV-2 protease was incubated for 10 min at 37 °C with treatments as follows: 40 mM DTT; 40 mM DTT with 241 nM (5 µg/ml) yeast MsrA; thioredoxin/thioredoxin reductase system (Trx/Tred) containing 100 µM NADPH, 5 µM thioredoxin and 3 µg/ml thioredoxin reductase; or Trx/Tred with 241 nM yeast MsrA. The relative specific activity was calculated as a percentage of the activity of unoxidized, untreated HIV-2 protease control run at the same time. As an additional control, unoxidized HIV-2 protease was also incubated in the presence of the different reducing reagents. In the presence of DTT the activity was 91% of the untreated control and in the presence of thioredoxin/thioredoxin reductase the activity was 104% of the untreated control.

Conditions			Protease activity restored (%)
DTT	Trx/Tred	MsrA	
+	—	—	0
+	—	+	17.8
—	+	—	0
—	+	+	22.3

MsrA partly restores enzyme activity of the oxidized form of HIV-2 protease by reduction of methionine sulfoxide 95

We previously demonstrated that the oxidation of Cys-95 of HIV-1 protease with glutathione could be reversed with the cellular enzyme thioltransferase. We hypothesized that MsrA, a cellular enzyme capable of reducing methionine residues, might similarly reverse the oxidation of the methionine residues in HIV-2 protease. To explore this possibility, we tested whether oxidized HIV-2 protease was a substrate for MsrA. Incubation of oxidized HIV-2 protease with the reducing agent DTT and increasing concentrations of MsrA led to a dose-dependent restoration of protease activity that reached a plateau close to 40% of the activity of the unoxidized protease control (Figure 3). Exposure of oxidized HIV-2 protease to concentrations of MsrA higher than 1 µM did not significantly improve the extent of restoration of protease activity (Figure 3). However, no restoration of protease activity was seen when DTT was used alone (Table 1). In addition, longer incubation times or additional MsrA added to the reaction mixture after the initial incubation did not further restore activity, suggesting that the failure to restore protease activity completely was not due to insufficient MsrA or to its inactivation in the reaction mixture (results not shown). In the cell, MsrA is believed to perform the reduction of methionine sulfoxide with the use of NADPH/thioredoxin/thioredoxin reductase as an enzyme regeneration system. The thioredoxin reductase system was able to replace DTT in the reaction mixture, resulting in a restoration of protease activity comparable to that obtained with DTT (Table 1). Under both conditions, the restoration of protease activity was dependent on the presence of MsrA (Table 1). These results suggest that oxidized HIV-2 protease can serve as a substrate for MsrA and can lead to partial restoration of protease activity.

Next we determined whether MsrA-dependent restoration of HIV-2 protease activity from the oxidized form of HIV-2 protease was due to the reduction of methionine sulfoxide 76 and/or methionine sulfoxide 95. Oxidized HIV-2 protease, before and after treatment with MsrA, was treated with trypsin and the resultant peptides were separated and analysed by RP-HPLC/MS. Trypsin cleavage of the oxidized HIV-2 protease followed by RP-HPLC/MS analysis revealed the predicted oxidized peptide fragments of 1622 amu (for residues 73–87) and 1275 amu (for residues 88–99) (Figure 4). Both peptides had a mass increase

Figure 4 RP-HPLC/MS analysis of peptides after digestion of the oxidized HIV-2 protease by trypsin with or without exposure to MsrA

The oxidized HIV-2 protease was treated for 10 min without or with 1.3 µM MsrA, as described. An aliquot of the sample was used to measure protease activity and the remainder was digested with trypsin. The peptides were analysed by RP-HPLC/MS. The extent of reduction of methionine sulfoxide to methionine was determined by normalizing the areas of the methionine-containing peptides to that of peptide 1–7 (unaffected by oxidation). Results are shown without MsrA (top panel) and with MsrA (bottom panel). The identities of the tryptic peptides of HIV-2_{rod} sequence as shown in Figure 1 are as follows: peak 1, p1–7 (904.4 amu); peak 2, p73–87⁺¹⁶ (1622.2 amu); peak 3, p46–72 (3017.8 amu); peak 4, p8–45 (4000.4 amu); peak 5, p88–99⁺¹⁶ (1274.5 amu); peak 6, p88–99 (1258.5 amu). The position of the reduced form of the Met-76-containing peptide is indicated. The expected retention time for the unoxidized Met-76-containing peptide was 21.5 min. MsrA reduced 32% of the peptide containing methionine sulfoxide 95, and 0% of the peptide containing methionine sulfoxide 76.

of 16 amu, which was consistent with the oxidation of the methionine residue in each peptide. Exposure of the oxidized HIV-2 protease to MsrA followed by peptide mapping demonstrated a decrease in the area corresponding to the peptide containing methionine sulfoxide 95 (Figure 4, peak 5) and an increase in the unoxidized form of this peptide (Figure 4, peak 6). Exposure to MsrA had no effect on the peptide containing methionine sulfoxide 76 (Figure 4, peak 2); the unoxidized form of that peptide was not detected (Figure 4). However, the unoxidized peptide containing residue 76 was readily detected after trypsin cleavage of the native unoxidized HIV-2 protease, indicating that the lack of detection of this peptide after treatment with MsrA was due to the inability of MsrA to reduce methionine sulfoxide 76. A quantitative analysis by RP-HPLC indicated that exposure of the oxidized HIV-2 protease to MsrA for 10 min reduced approx. 32% of the methionine sulfoxide 95 back to methionine while restoring 25% of the protease activity. This indicates that the selective reduction of methionine sulfoxide 95 of HIV-2 protease by MsrA is coupled to the restoration of protease activity.

Figure 5 RP-HPLC chromatograms for C67A C95A and C67A C95M HIV-1 proteases before and after exposure to H₂O₂ and the effect on protease activity

C67A C95A (upper panel) or C67A C95M (lower panel) HIV-1 proteases were incubated for 10 min at 37 °C in the absence (solid lines) or presence (broken lines) of 150 mM H₂O₂ in 20 mM HCl; they were then analysed by RP-HPLC or for protease activity (insets). The peaks corresponding to the protease activity were collected and aliquots were subjected to ES-MS analysis to determine the predominant molecular mass for each peak. The change in molecular mass [in amu (MU)] obtained for the untreated and treated proteases is shown above each peak.

C67A C95M HIV-1 protease is reversibly inactivated by the oxidation of Met-95

Previously it was shown that thiol-oxidizing agents or glutathiolation could not inactivate a mutant form of HIV-1 protease lacking both cysteine residues at positions 67 and 95 [19,25]. In addition, this mutated protease was not inactivated by exposure to H₂O₂ (results not shown). Thus the oxidative inactivation of HIV-1 protease was dependent on the presence of the cysteine residues; further studies showed that the critical residue for oxidative inactivation was at position 95 [19]. We therefore decided to examine whether inserting a methionine residue at position 95 in the HIV-1 protease mutant normally resistant to oxidative inactivation would be sufficient to restore susceptibility to H₂O₂, like that observed for HIV-2 protease. Exposure of the resistant form of the HIV-1 protease, C67A C95A, to H₂O₂ under the same conditions as described for HIV-2 protease resulted in the generation of earlier-eluting forms of the protease as determined by RP-HPLC (Figure 5, upper panel). RP-HPLC/MS analysis of Lys C digests of these modified forms of the protease indicated a mass increase of 16 and 32 amu as a result of the oxidation of Met-36 and/or Met-46 in the protease (results not shown). However, oxidation of these two surface-exposed methionine residues did not affect the activity of C67A C95A protease (Figure 5, inset to upper panel). In contrast,

Figure 6 Effect of H₂O₂ on viral polyprotein processing of immature HIV-1 wild-type, HIV-1 C67A C95A mutant or HIV-2 wild-type virus particles after the removal of HIV-1 protease inhibitor

Immature virus particles were incubated in the presence of the indicated concentrations of H₂O₂ at 37 °C for 6 h. The first lane (U) for each virus represents the unprocessed virus preparation after the removal of the protease inhibitor. Protease activity was stopped by the addition of SDS sample buffer. Blots were probed with a monoclonal anti-p24 antibody or polyclonal antibody against p26. The migrations of the unprocessed p55 or p57 gag polyproteins and the processed p24/25 or p26 mature capsid proteins are indicated. (A) Immature HIV-1 wild-type preparation analysed by Western blot after processing for 6 h after the removal of protease inhibitor and treatment with increasing concentrations of H₂O₂; (B) immature HIV-1 C67A C95A viral preparation treated as described in (A); (C) immature HIV-2 wild-type viral preparation treated as described in (A).

C67A C95M HIV-1 protease was inactivated when exposed to H₂O₂ (Figure 5, inset to lower panel). Inactivation of C67A C95M protease with H₂O₂ coincided with the accumulation of a protease species as detected by RP-HPLC containing a mass increase of 48 amu, which was consistent with the oxidation of all three methionine residues (residues 36, 46 and 95) to methionine sulfoxide (Figure 5, lower panel). To a smaller degree, two other forms also detected by RP-HPLC were produced with increases of 16 and 32 amu. The complete inactivation of the enzyme after this treatment (Figure 5, inset to lower panel) was consistent with the oxidation of Met-95 in all three forms of the protease. Both wild-type HIV-1 protease (containing both cysteine residues) and the C67A C95M HIV-1 protease were also inactivated by H₂O₂ treatment in the standard protease assay pH of 5.5 (95% and 75% decreases in activity in comparison with untreated controls), whereas the C67A C95A protease activity decreased by only 17%. This demonstrates that a cysteine or methionine residue at position 95 in the dimer interface of the protease is sufficient to give rise to an enzyme susceptible to oxidative inactivation even under conditions near physiological pH.

Exposure of the inactive oxidized form of HIV-1 C67A C95M protease to 1.3 μM MsrA was able to restore approx. 40% of the original HIV-1 protease activity. This compared with a 38% restoration of activity for oxidized HIV-2 protease run under identical conditions. RP-HPLC/MS analysis of proteolytic

digests indicated that MsrA reduced the peptide containing methionine sulfoxide 95 to the unoxidized form; the extent of reduction of methionine sulfoxide 95 paralleled the restoration of protease activity (results not shown).

H₂O₂ inhibits HIV-1 and HIV-2 viral polyprotein processing

We reported recently that the exposure of released immature HIV-1 viral particles to H₂O₂ results in the inhibition of post-release polyprotein processing and that this effect requires the presence of Cys-95 in the protease [23]. It was therefore of interest to determine whether H₂O₂ would inhibit HIV-2 polyprotein processing in released particles. To evaluate this, immature HIV-2, HIV-1 and C67A C95A HIV-1 particles were isolated in the presence of the protease inhibitor KNI-272, as described previously for HIV-1 [23]. After the protease inhibitor had been washed from the immature virus particles, the particles were incubated in the presence of increasing concentrations of H₂O₂ in conditioned medium (pH 6.0) at 37 °C for 6 h and left to undergo polyprotein processing. Polyprotein processing of the immature wild-type HIV-1 viral particles *ex vivo* was decreased after exposure to 100 µM H₂O₂ as evidenced by a 75 % decrease in p25/p24 accumulation, based on densitometry, and a corresponding increase in the p55 gag (group-specific antigen) precursor (Figure 6A). In contrast, the p25/24 accumulation was decreased by only 6 % when the C67A C95A HIV-1 virus was exposed to 100 µM H₂O₂ (Figure 6B). This provides evidence that the inhibition of polyprotein processing by H₂O₂ is due primarily to oxidation of the cysteine residues of HIV-1 protease. HIV-2 viral polyprotein processing of the immature viral particles was also inhibited by exposure to H₂O₂ in a dose-dependent fashion, as indicated by a decrease in the mature p26 capsid protein and a corresponding increase in p57 gag and its intermediates (Figure 6C). However, the effect of H₂O₂ on HIV-2 polyprotein processing was rather less than that for HIV-1 with a decrease in p26 accumulation of 60 % at 500 µM H₂O₂. Taken together, these results suggest that oxidation of either HIV-1 or HIV-2 virus particles can result in the inhibition of viral polyprotein processing due to oxidation of the protease.

DISCUSSION

We have shown that HIV-2 protease can be reversibly inactivated after oxidation of the methionine residue at position 95, which is highly conserved among the sequences of the HIV-2 protease. This mechanism of inactivation is related to, yet distinct from, that previously demonstrated for the reversible oxidation of Cys-95 of the HIV-1 protease, also a highly conserved residue [19]. Interestingly, the oxidation and inactivation of both enzymes are reversible with a cellular enzyme, thioltransferase to reverse glutathiolation of HIV-1 protease, whereas MsrA can reduce the methionine oxidation of HIV-2 protease. In addition, both enzymes preferentially reduce residue 95 of the protease after its oxidation. Initially, this preference for reduction of residue 95 over that for residue 76 was surprising on the basis of the buried nature of residue 95 in the enzyme's crystal structure [26]. However, after oxidation of the protease there would probably be a decrease in the dimer's affinity owing to the addition of a hydrophilic group in the dimer interface resulting in exposure of residue 95. These studies support a possible role for thioltransferase and MsrA in maintaining or regulating the function of the protease in the HIV life cycle after oxidative stress. Although future studies will be required to address this point directly, our previous studies indicating that the reducing agent DTT increased the post-release processing of HIV-1 particles

indicates that a portion of the HIV-1 protease is oxidized in immature virions released from infected cells. This indicates that the protease is susceptible to oxidative inactivation *in vivo* [23].

Treatment of the oxidized HIV-2 protease with MsrA restored protease activity only partly (approx. 40 %), yet this paralleled the extent to which residue 95 was reduced back to methionine. The most likely explanation for the limited restoration of protease activity is related to the ability of MsrA to reduce only the *S* diastereoisomer of methionine sulfoxide in proteins, as recently demonstrated for calmodulin [27]. The exposure of the protease to H₂O₂ *in vitro* would be expected to generate approximately equal amounts of the *R* and *S* forms of methionine sulfoxide at residue 95. If this were so, it would only be possible to restore a maximum of 50 % of the protease activity if all of the *S* sulfoxide were reduced after treatment with MsrA.

The specific role of the oxidizable residues at position 95 of HIV-1 and HIV-2 protease is unknown. The highly conserved nature of these residues, along with the information that this residue is expendable for protease activity [19,28], suggests that their presence might be advantageous to the viral life cycle. It is conceivable that these residues serve to prevent the premature activation of protease in the cytoplasm or decrease its activity in cells under oxidative stress. In this regard, Met-95 of HIV-2 protease is perhaps more susceptible to oxidation in the gag-pol (where pol is polymerase) polyprotein, in which it would be more exposed than in the active protease dimer. These residues might also provide specific targets within the dimer interface for the development of compounds designed to inhibit protease activity irreversibly through the disruption of dimerization. It is interesting to note that the protease sequences of SIV, the virus from which HIV-1 and HIV-2 is thought to have evolved [29], contains either a cysteine or a methionine residue at the dimer interface [24]. It is also interesting that other proteases, including those from feline immunodeficiency virus and Rous sarcoma virus, also contain oxidizable amino acids at the dimer interface; we are currently exploring the possibility that specific oxidative inactivation of these retroviral proteases might occur. This work should be useful as a model for the continued study of the redox regulation of viral and cellular dimeric enzymes whose function might also be altered under different redox states.

We thank Keisuke Yusa and Hiroaki Mitsuya for the use of HIV-1 molecular clones, and Barbara Berlett for amino acid analysis. Funding for this study was provided in part by an award from the AIDS Targeted Antiviral Program at NIH.

REFERENCES

- Chai, Y.-C., Ashraf, S. S., Rokutan, K., Johnston, R. B. J. and Thomas, J. A. (1994) Arch. Biochem. Biophys. **310**, 273–281
- Ravichandran, V., Seres, T., Moriguchi, T., Thomas, J. A. and Johnston, Jr., R. B. (1994) J. Biol. Chem. **269**, 25010–25015
- Rokutan, K., Johnston, R. B. J. and Kawai, K. (1994) Am. J. Physiol. **266**, G247–G254
- Bandyopadhyay, S., Starke, D. W., Mieyal, J. J. and Gronostajski, R. M. (1998) J. Biol. Chem. **273**, 392–397
- Davis, D. A., Newcomb, F. M., Starke, D. W., Ott, D. E., Mieyal, J. M. and Yarchoan, R. (1997) J. Biol. Chem. **272**, 25935–25940
- Gravina, S. A. and Mieyal, J. J. (1993) Biochemistry **32**, 3368–3376
- Terada, T., Nishimura, M., Oshida, H., Oshida, T. and Mizoguchi, T. (1993) Biochem. Mol. Biol. Int. **29**, 1009–1014
- Terada, T. (1997) Int. J. Biochem. Cell Biol. **29**, 985–992
- Yoshitake, S., Nanri, H., Fernando, M. R. and Minakami, S. (1994) J. Biochem. (Tokyo) **116**, 42–46
- Wang, G. M., Wu, F., Raghavachari, N. and Reddan, J. R. (1998) Exp. Eye Res. **66**, 477–485
- Raghavachari, N., Qiao, F. and Lou, M. F. (1999) Exp. Eye Res. **68**, 715–724
- Brot, N. and Weissbach, H. (1983) Arch. Biochem. Biophys. **223**, 271–281
- Brot, N. and Weissbach, H. (1991) Biofactors **3**, 91–96

- 14 Vogt, W. (1995) Free Radicals Biol. Med. **18**, 93–105
- 15 Giorba, M. A., Heinemann, S. H., Weissbach, H., Brot, N. and Hoshi, T. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 9932–9937
- 16 Moskovitz, J., Berlett, B. S., Poston, J. M. and Stadtman, E. R. (1999) Methods Enzymol. **300**, 239–244
- 17 Moskovitz, J., Berlett, B. S., Poston, J. M. and Stadtman, E. R. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 9585–9589
- 18 Wizemann, T. M., Moskovitz, J., Pearce, B. J., Cundell, D., Arvidson, C. G., So, M., Weissbach, H., Brot, N. and Masure, H. R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 7985–7990
- 19 Davis, D. A., Dorsey, K., Wingfield, P. T., Stahl, S. J., Kaufman, J., Fales, H. M. and Levine, R. L. (1996) Biochemistry **35**, 2482–2488
- 20 Levine, R. L., Mosoni, L., Berlett, B. S. and Stadtman, E. R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 15036–15040
- 21 Moskovitz, J., Weissbach, H. and Brot, N. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 2095–2099
- 22 Apfel, A., Fischer, S., Goldberg, G., Goodley, P. C. and Kuhlmann, F. E. (1995) J. Chromatogr. A **712**, 177–190
- 23 Davis, D. A., Yusa, K., Gillim, L. A., Newcomb, F. M., Mitsuya, H. and Yarchoan, R. (1999) J. Virol. **13**, 1156–1164
- 24 Human retroviruses and AIDS 1997: a Compilation and Analysis of Nucleic Acid and Amino Acid Sequences (Korber, B., Hahn, B., Foley, B., Mellors, J. W., Leitner, T., Myers, G., McCutchan, F. and Kuiken, C. L., eds.), Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, NM, U.S.A
- 25 Davis, D. A., Branca, A. A., Pallenberg, A. J., Marschner, T. M., Patt, L. M., Chatlynne, L. G., Humphrey, R., Yarchoan, R. and Levine, R. L. (1995) Arch. Biochem. Biophys. **322**, 127–134
- 26 Fitzgerald, P. M., McKeever, B. M., VanMiddlesworth, J. F., Springer, J. P., Heimbach, J. C., Leu, C. T., Herber, W. K., Dixon, R. A. and Darke, P. L. (1990) J. Biol. Chem. **265**, 14209–14219
- 27 Sharov, V. S., Ferrington, D. A., Squier, T. C. and Schoneich, C. (1999) FEBS Lett. **455**, 247–250
- 28 Karlstrom, A. R. and Levine, R. L. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5552–5556
- 29 Fukasawa, M., Miura, T., Hasegawa, A., Morikawa, S., Tsujimoto, H., Miki, K., Kitamura, T. and Hayami, M. (1988) Nature (London) **333**, 457–461
- 30 Sayle, R. A. and Milner-White, E. J. (1995) Trends Biochem. Sci. **20**, 374

Received 13 September 1999/8 November 1999; accepted 8 December 1999