Inhibition of Human Cytomegalovirus UL80 Protease by Specific Intramolecular Disulfide Bond Formation†

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ABSTRACT: A symmetrically substituted disulfide compound, CL13933, was identified as a potent inhibitor of human cytomegalovirus UL80 protease. Two types of inhibited protease were observed, depending on inhibitor concentration. At high concentrations, CL13933 formed a covalent adduct with the protease on Cys residues. At lower concentrations, this compound induced specific intramolecular disulfide formation between Cys84 and Cys87, and between Cys138 and Cys161. In contrast, Cys202 did not form disulfide bonds. Inhibition was reversed upon reduction of the protease. Each of the five cysteines of the UL80 protease was individually mutated to Ala. Each of the mutant proteases retained enzymatic activity, but mutants C138A and C161A were resistant to inhibition by CL13933, suggesting that disulfide bond formation between Cys138 and Cys161 is responsible for inhibition. This disulfide is apparently not induced by air oxidation. Examination of the CL13933 loading patterns of wild type and the five mutant proteases by mass spectrometry revealed that residues Cys87, Cys138, and Cys161 react with CL13933, and that the disulfide pair partner of each (Cys84, Cys161, and Cys138, respectively) is able to displace the compound via thiol−disulfide exchange. The possible significance of these reactive thiols in the protease is discussed.

Human cytomegalovirus (HCMV)[1] is a betaherpesvirus which can cause severe illness or death of immunocompromised individuals, such as AIDS patients or recipients of organ and bone marrow transplants (Alford & Britt, 1990). The 28-kDa protease encoded by the UL80 gene is essential for viral replication, based on studies of its homolog from herpes simplex virus type 1, UL26 (Preston et al., 1983; Gao et al., 1994). Such proteases have been identified in all herpesviruses sequenced to date (Liu & Roizman, 1991; Welch et al., 1993) and appear to function late in viral maturation to cleave the viral assembly protein, an event which is coupled to viral DNA encapsidation (Preston et al., 1992; Rixon et al., 1988). These proteases are translated from larger precursors which undergo autoproteolytic cleavage. In particular, the 85-kDa UL80 protease precursor is cleaved at the maturation site (after amino acid 643, which is identical to the assembly protein cleavage site), at the release site (after amino acid 256, releasing the 28-kDa protease), and at two internal sites (after amino acids 143 and 209) which are within the 28-kDa protease itself; site 209 is recognized in mammalian cells but not in *Escherichia coli* (Welch et al., 1991, 1993; Baum et al., 1993; Jones et al., 1994). Wild-type 28-kDa HCMV protease expressed in *E. coli* is a mixture of the intact 28-kDa protease and the 16- and 13-kDa proteins which result from cleavage at site 143 (Baum et al., 1993). It has recently been demonstrated that the 16- and 13-kDa proteins can associate noncovalently, and that this "two-chain" protease is enzymatically active *in vitro* (Holwerda et al., 1994).

Various studies suggest that cytomegalovirus protease is a member of the serine protease class. These include inhibition by serine-specific reagents (Liu & Roizman, 1992), mutagenesis (Welch et al., 1993), and, most recently, labeling of the putative active site Ser132 with diisopropylfluorophosphate (Holwerda et al., 1994; DiIanni et al., 1994; Stevens et al., 1994). However, we and others have noted that HCMV protease is also sensitive to Cys-specific reagents (Baum et al., 1993; Burck et al., 1994).

In this study, we report that HCMV protease is inhibited by a Cys-specific compound, CL13933. Two types of inhibited protease were observed by mass spectrometry, depending on inhibitor concentration. At high concentrations, CL13933 formed a covalent adduct with the protease on Cys residues. At lower concentrations, CL13933 induced formation of specific intramolecular disulfides, by a thiol−disulfide exchange reaction. Mutational analysis of HCMV protease was used to identify the reactive thiols and the inhibitory disulfide. The possible biological significance of the reactive thiols in the protease is discussed.

MATERIALS AND METHODS

Protease Constructs. Wild-type 28-kDa HCMV protease, encoded by p30k, has been described previously (Baum et al., 1993). Mutant A144L does not autodigest into the 16- and 13-kDa cleavage products but is otherwise similar to wild type (Welch et al., 1993; Jones et al., 1994; Holwerda

† We dedicate this paper to the memory of Yasha Gluzman, our colleague, mentor, and friend.

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[1] Abbreviations: HCMV, human cytomegalovirus; IAM, iodoacetamide; BMS, bismercaptoethyl sulfone; DTT, dithiothreitol; SPA, scintillation proximity assay.

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et al., 1994). Single-stranded site-directed mutagenesis (Sambrook et al., 1989) was performed to individually substitute Ala for Cys at positions 84, 87, 138, 161, and 202.

Protease Purification. *E. coli* BL21(DE3)pLysS harboring wild-type or mutant protease under phage T7 promoter control were induced as described previously (Baum et al., 1993), lysed with a French press (SLM Instruments, Inc., 1400 psig), and fractionated by centrifugation (30 min, 4 °C, 24000*g*) into the pellet (containing inclusion bodies) and supernatant. Protease was solubilized from inclusion bodies with urea (Baum et al., 1993) and used directly after dialysis. Protease concentration was determined by Bio-Rad protein assay or by amino acid analysis.

Protease Assay. A scintillation proximity assay (SPA) (Bosworth & Towers, 1989; Brown et al., 1994) was used to monitor HCMV protease activity and is described in detail elsewhere (Baum et al., 1996). The peptide substrate contains the assembly protein cleavage site, GVVNASARL, and is biotinylated on the N-terminus and radiolabeled on the C-terminus. Cleavage of the peptide by the protease liberates the radiolabel from the biotin, resulting in a decrease in radioactive signal upon capture with streptavidin-coated SPA beads. In general, HCMV protease (2 μM) was preincubated with potential inhibitors for 2 h at 25 °C in 10 mM Tris-Cl, pH 7.5/50 mM NaCl. Substrate was added and incubation continued for 2 h. Reactions were terminated with pH 5.5 stop buffer containing streptavidin-coated SPA beads (Amersham Corp.) followed by scintillation counting. "Minus protease" (−P) and "plus protease" (+P) controls were used to calculate percent inhibition from counts per minute as follows:

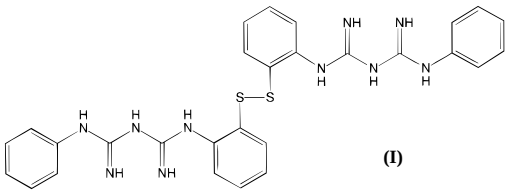
$$\% \text{ inhibition} = [\text{sample} - (+\text{P})]/[(-\text{P}) - (+\text{P})] \times 100$$

Mass Spectrometry. Electrospray ionization mass spectra were obtained with a VG Quattro triple quadrupole mass spectrometer equipped with a VG electrospray source, rf hexapole lens, and Megaflow gas nebulizer probe. HCMV protease samples were diluted to ~10 pM/μL in 35% acetonitrile/3% acetic acid prior to injection. Ten to twenty spectra were averaged, smoothed, baseline subtracted, centroided, and transformed from a mass/charge (*m*/*z*) axis to a mass axis.

Peptide Mapping and Disulfide Identification. A144L HCMV protease was incubated with or without a 9-fold molar excess of CL13933 in 10 mM Tris-Cl, pH 7.5/50 mM NaCl (2 h at 22 °C), followed by a Sephadex G-25 spin column (Maniatis et al., 1982). Samples were denatured in 6 M urea/50 mM Tris-Cl, pH 7.5, and alkylated (carboxyamidomethylated) with iodoacetamide (IAM) (15 mM, 15 min at 22 °C). After a second spin column, samples were digested with trypsin (5 h at 37 °C) in 1 M urea/50 mM Tris-Cl, pH 7.5/0.5% hydrogenated Triton X-100. Digestion was stopped with 100 μM *N*-tosyl-L-lysine chloromethyl ketone, and half of each sample was reduced with dithiothreitol (DTT) (1 mM, 15 min at 50 °C). Peptide digests were subjected to reverse-phase HPLC (Hewlett Packard model 1090) using an Aquapore OD300 C18 column (ABI). Selected peptides were analyzed by N-terminal amino acid sequencing (ABI model 477A protein sequencer) and mass spectrometry.

RESULTS

Identification of CL13933 as a Potent, Irreversible Inhibitor of HCMV Protease. By randomly screening a chemical library, compound CL13933 (1,1′-(dithio-di-*o*-phenylene)-*bis*-(5-phenylbiguanide) (**I**), a symmetrically substituted



(I)

disulfide of molecular mass 568 Da, was identified as a potent inhibitor of wild-type HCMV protease, with IC_{50} = 5 μM. The protease is present in this assay at ~2 μM; the requirement of relatively high protease concentration for cleavage of peptide substrate (DiIanni et al., 1994; Burck et al., 1994; Sardana et al., 1994) or assembly protein precursor (Baum et al., 1993) is typical for herpesvirus proteases. Wild-type protease is capable of internal cleavage between amino acids 143 and 144, generating 16- and 13-kDa cleavage products (amino acids 1−143 and 144−256, respectively) (Baum et al., 1993). Protease A144L, which fails to generate the 16- and 13-kDa cleavage products but is otherwise similar to wild type (Jones et al., 1994), also displays an IC_{50} of 5 μM. When 4 μM HCMV protease was used in the assay, the observed IC_{50} of CL13933 likewise doubled, to 10 μM. Thus, complete inhibition of the protease was achieved by approximately a 5-fold molar excess of the inhibitor over the protease under these conditions. This inhibition was found to be irreversible, as defined by the failure to recover enzymatic activity upon removal of the free compound either by gel filtration using spin columns or by dialysis.

Mechanism of Inhibition: Covalent Adduct versus Disulfide Bond Formation. Since CL13933 contains a disulfide bond, this suggested that the compound's mechanism of action could be via reaction with the Cys residue(s) of HCMV protease. To determine whether an adduct is formed between HCMV protease and CL13933, as evidenced by an increase in protease molecular mass, reactions containing the protease and various amounts of the inhibitor were prepared, assayed for inhibition, and analyzed by mass spectrometry (Figure 1). The conditions for mass spectrometry are sufficiently denaturing that only covalent adducts, and not noncovalently bound complexes, are expected to be detected. The observed mass of protease A144L is 28 080 Da (Figure 1A), in excellent agreement with the calculated mass (28 083 Da). Protease of 27 948 Da lacking N-terminal Met is also observed (Baum et al., 1993), due to processing by *E. coli* Met aminopeptidase (Ben-Bassat et al., 1987). Shoulder peaks which are ~43 Da greater in mass than these main peaks (e.g., the 28 123 Da shoulder of the 28 080 Da peak, Figure 1A) arise from urea-induced carbamylation (Stark et al., 1960) of the protease during preparation from *E. coli* (Materials and Methods). The shoulder peaks are absent if the protease is prepared in 6 M guanadine hydrochloride instead of urea (data not shown). At a 20-fold molar excess of CL13933, the protease is fully inhibited, and 60% of the protease molecules undergo an increase in mass of 284 Da (28 364 Da, panel D). This mass is consistent with addition of a half molecule of CL13933 to HCMV protease. Twenty

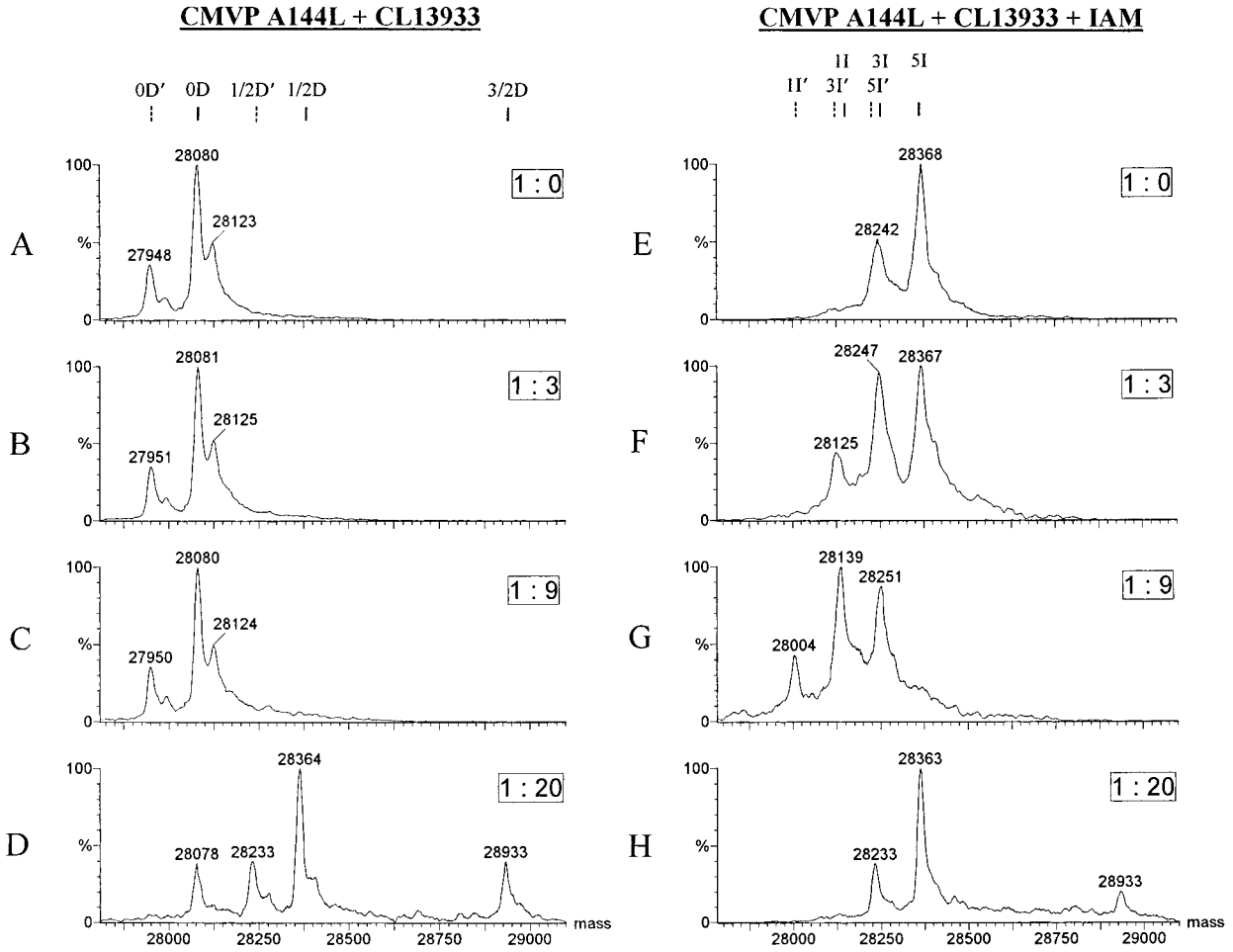


FIGURE 1: Transformed electrospray ionization mass spectra of A144L HCMV protease incubated with CL13933. Protease (50 μM) was incubated without and with CL13933 in 10 mM Tris-Cl, pH 7.5/50 mM NaCl (2 h at 25 °C). An aliquot of each reaction was then diluted and assayed in the SPA at 2 μM final protease concentration; samples A, B, C, and D displayed 0, 40, 100, and 90% inhibition, respectively. Samples were desalted and excess CL13933 removed by spin column chromatography (Maniatis et al., 1982) prior to mass spectrometry (panels A−D). Panel A, no CL13933. Panels B, C, and D: 3-, 9-, or 20-fold molar excess of CL13933. For alkylation studies (panels E−H), control and CL13933-treated samples were denatured in 6 M urea and incubated with 15 mM IAM (45 min at 25 °C), followed by an additional spin column and mass spectrometry. Panel E, no CL13933. Panels F, G, and H: 3-, 9-, or 20-fold molar excess of CL13933. The masses of zero, one half, and three halves CL13933 adducts are indicated by 0D, 1/2D, and 3/2D, respectively. The masses of one, three, and five alkyl groups are indicated by 1I, 3I, and 5I, respectively. Peaks labeled with a prime (′) are lacking the amino terminal Met and behave exactly as their counterparts containing Met. The shoulder peaks which are ~43 Da greater than their corresponding main peaks (e.g., the 28 125 Da peak in panels A, B, and C) result from carbamylation of the protease during preparation in urea (see text).

percent of the protease molecules show no adducts, and 20% undergo addition of 853 Da (28 933 Da, panel D), consistent with the addition of 1.5 molecules (or three halves) of CL13933 to HCMV protease.

Inhibition of HCMV protease is prevented if CL13933 is pretreated with the reducing agent DTT (data not shown). This information, coupled with the observation that the adduct on the protease corresponds to multiples of one half of the mass of CL13933, suggests that Cys(s) on HCMV protease attack a sulfur of the disulfide bond of CL13933, resulting in the formation of a protease−CL13933 mixed disulfide.

Unexpectedly, at concentrations of CL13933 which failed to produce a covalent adduct (e.g., 3- and 9-fold molar excess of compound over protease, Figure 1B,C), significant inhibition (40% and 100%, respectively) of HCMV protease was observed. Evidence that HCMV protease formed intramolecular disulfide bonds, and that disulfide bond formation could be responsible for inhibition of the enzyme, was provided by examining wild-type HCMV protease by reducing and nonreducing SDS−PAGE, after incubation with CL13933 (Figure 2). For the control sample, the presence or absence of the reducing agent β-mercaptoethanol during sample boiling makes no difference, in that approximately equal amounts of 28-, 16-, and 13-kDa species are observed (lanes 1 and 2). In contrast, the CL13933-treated sample lacking reducing agent (lane 3) contains protein which migrates mainly at 28 kDa; the intensity of the 16- and 13-kDa proteins is greatly diminished compared to the control samples (lanes 1 and 2). This 28-kDa species is likely to be a mixture of authentic 28-kDa protein (with internal disulfides and/or covalently attached CL13933, since this species migrates slightly faster than fully reduced 28-kDa protein, compare lanes 3 and 4) and also 16-kDa protein which is disulfide bonded to 13-kDa protein and therefore migrates as a 28-kDa protein. When β-mercaptoethanol is present, the CL13933-treated sample shows the control pattern of equal amounts of 28-, 16-, and 13-kDa species (lane 4), demonstrating that disulfide bond formation between cysteines on the 16- and 13-kDa proteins occurs in the presence of CL13933.

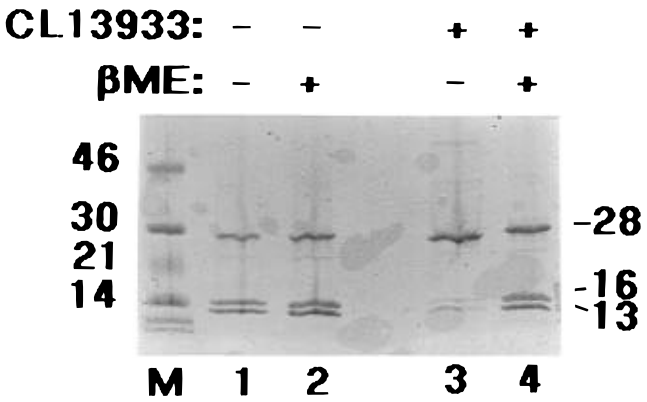


FIGURE 2: Electrophoresis of CL13933-treated HCMV protease under reducing and nonreducing conditions. Wild-type HCMV protease samples incubated without or with 10-fold molar excess of CL13933 (which displayed 90% inhibition in the SPA) were diluted with SDS sample buffer containing or lacking the reducing agent β-mercaptoethanol (BME) as indicated. After boiling, samples were applied to a 10−15% gradient Phast Gel (Pharmacia), and proteins were visualized with Coomassie Blue. The positions of molecular weight markers (M), and the 28-kDa protease and the 16- and 13-kDa internal cleavage products, are indicated.

Counting of Free Cysteines in CL13933-Treated HCMV Protease. Five cysteines (Cys84, Cys87, Cys138, Cys161, and Cys202) are present in wild-type HCMV protease. A loss of 2 Da from the 28 080 Da A144L protease, which is expected for each disulfide bond formed, cannot be resolved by quadrupole mass spectrometry. To determine whether (and how many) disulfide bonds were formed on HCMV protease as a result of incubation with CL13933, free cysteines were "counted" using the Cys-specific alkylating agent iodoacetamide (IAM) (Means & Feeney, 1971). An increase in mass of 57 Da is expected for each Cys residue which reacts with IAM. Aliquots of the control and CL13933-treated protease samples shown in Figure 1 (panels A−D) were denatured, alkylated, and analyzed by mass spectrometry to determine the number of alkyl groups added (panels E−H). As shown in Figure 1E (peaks 5I and 5I′), most of the control protease molecules have all five cysteines accessible to alkylation, indicating no disulfides are present, consistent with previous data (Burck et al., 1994). Denaturation of HCMV protease in urea is required for all five cysteine residues to react with IAM: in preliminary alkylation experiments without urea, only two cysteines were alkylated; with 4 M urea, only three cysteines were alkylated (data not shown).

Treatment of HCMV protease with a 9-fold molar excess of CL13933 (a concentration which fully inhibits but does not cause formation of an adduct between the compound and the protease, Figure 1C) results in substantial disulfide bond formation on HCMV protease: 60% of the HCMV protease molecules added only one alkyl group, indicating two disulfides were formed, and 40% of the HCMV protease molecules added three alkyl groups, indicating that one disulfide formed (Figure 1G). Since this protease sample is fully inhibited, these data suggest that one disulfide is sufficient to inhibit the protease. The extent of disulfide bond formation is dependent upon the concentration of CL13933 used: at 3-fold molar excess of CL13933, which caused only partial (40%) inhibition of protease activity, 40% and 20% of the protease contain one and two disulfide bonds, respectively, while 40% of the protease is fully reduced (able to add five alkyl groups) (Figure 1F).

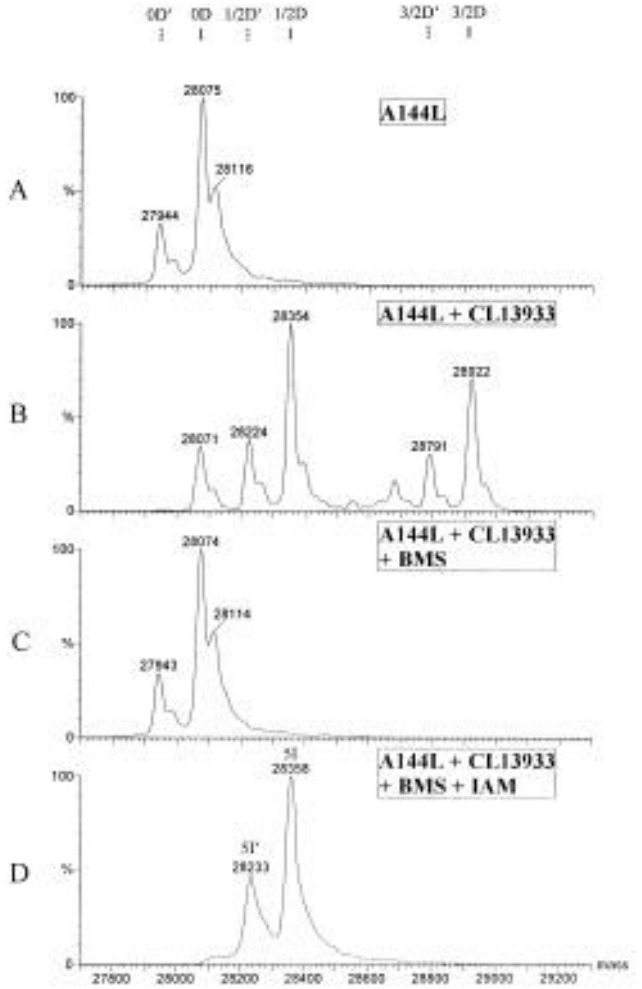


FIGURE 3: Reversal of CL13933 adducts and internal disulfides of HCMV protease by treatment with the reducing agent BMS. A144L HCMV protease (75 μM) incubated without (panel A) and with CL13933 (1.1 mM for 2 h) (panel B) was subjected to a spin column and mass spectrometry. A portion of the CL13933-treated sample was then reduced with BMS (15 mM, 15 min), followed by a spin column and mass spectrometry (panel C). A portion of the CL13933-treated, BMS-treated sample was then alkylated with IAM (see Figure 1) followed by a spin column and mass spectrometry (panel D). Samples A, B, and C displayed 0, 100, and 20% inhibition, respectively, upon testing at 2 μM final protease concentration in the SPA.

At 20-fold molar excess of CL13933 over protease, analysis of free cysteines becomes more complicated, because only those protease molecules which did not react covalently with CL13933 (20% of the total) are accessible for alkylation; also, the mass of HCMV protease with five alkyl groups (28 358 Da) cannot be distinguished from HCMV protease conjugated to one half CL13933. Nevertheless, it is clear that the protease molecules already conjugated with CL13933 (60% and 20% containing one half and three halves of CL13933, respectively) failed to undergo alkylation with IAM, suggesting that at least 80% of the total protease molecules have no free cysteines (Figure 1H). These data imply that the majority of cysteines not attached to CL13933 are in disulfide bonds and protected from alkylation.

Reversal of Inhibition by Treatment with Reducing Agent. To further support the hypothesis that disulfide bond formation is the cause of inhibition of HCMV protease, control and CL13933-treated A144L protease samples were subjected to treatment with the reducing agent bismercaptoethyl

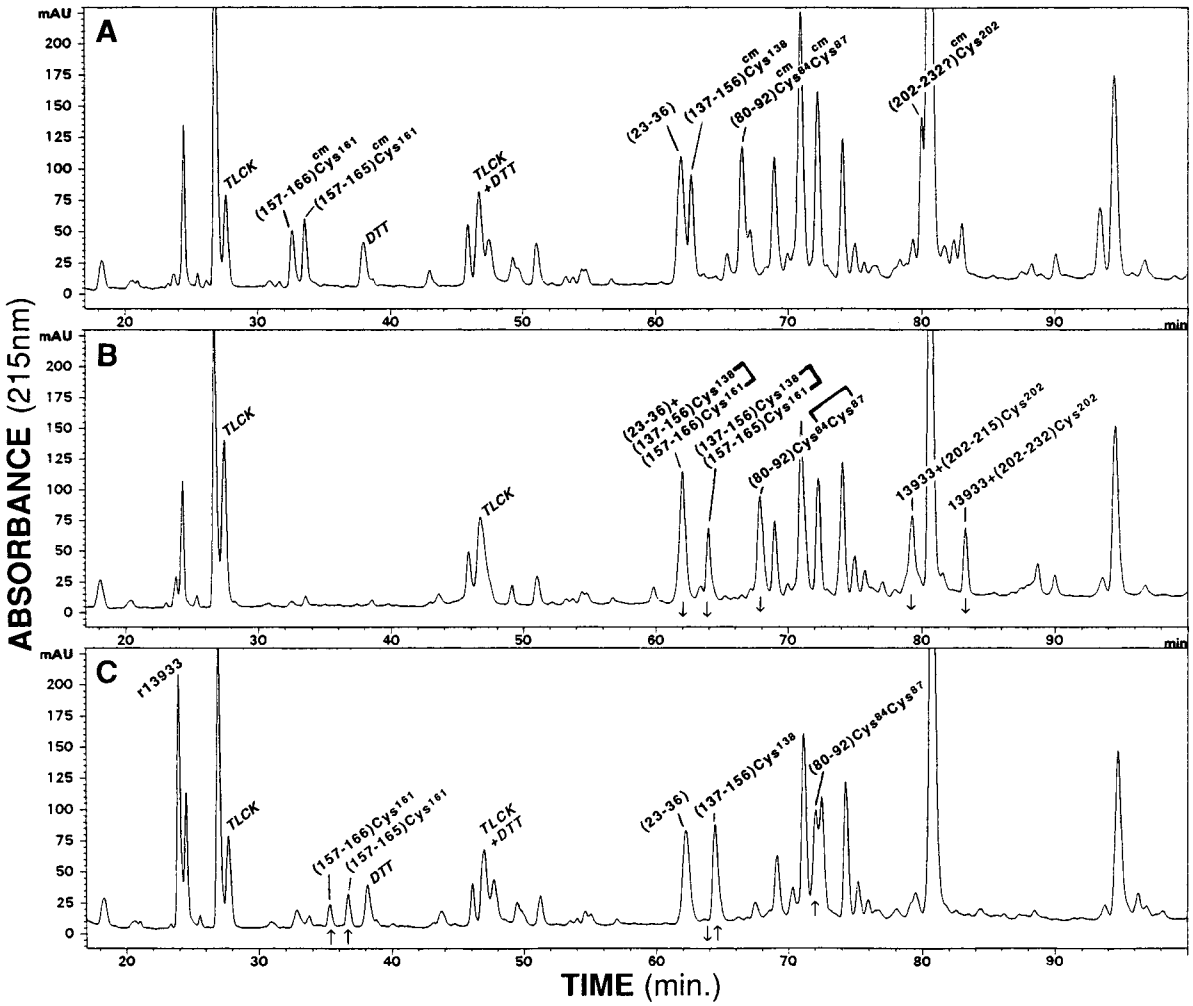


FIGURE 4: Separation of tryptic peptides of control and CL13933-treated HCMV protease, and identification of disulfide pairs. (A) Tryptic peptides from control, carboxyamidomethylated A144L HCMV protease were subjected to reverse-phase HPLC and eluted with a gradient of acetonitrile (0−8% in 10 min, to 36% in 80 min, to 80% in 15 min at 100 μL/min) in 0.05% trifluoroacetic acid. The DTT-reduced map is shown; the nonreduced map is identical. Numbers in parentheses refer to the first and last amino acid residues of a given peptide; carboxyamidomethylcysteine (Cyscm) residues are shown. (B) Tryptic peptides from CL13933-treated HCMV protease. Disulfide-linked peptides are shown by bars connecting Cys residues. Peptides containing one half molecule of CL13933 are indicated. (C) Tryptic peptides from CL13933-treated HCMV protease, following treatment with DTT. The reduced, free sulfhydryl-containing peptides (Cys#), reduced CL13933 (rCL13933), and peaks introduced by DTT or the trypsin inhibitor TLCK are indicated. Arrows denote the disappearance (↓) and appearance (↑) of peptides upon DTT treatment.

sulfone (BMS), which is a more effective reducing agent than DTT at neutral pH (Singh & Whitesides, 1994). Samples were tested for enzymatic activity and for adductand disulfide bond formation (Figure 3). Prior to BMS treatment, the CL13933-treated sample displayed complete (100%) inhibition; 50% of the protease molecules contained one half, and 30% contained three halves, of covalently attached CL13933 (Figure 3B). Alkylation patterns of the control and CL13933-treated samples were essentially identical to those shown in Figure 1, panels E and H, respectively. BMS treatment removed all adducts of CL13933 from the protease (Figure 3C). Also, BMS treatment fully reduced the cysteines, as demonstrated by addition of five alkyl groups upon treatment with IAM (Figure 3D). The CL13933-treated protease recovered nearly complete activity, going from 100% inhibition to only 20% inhibition after BMS treatment. We cannot explain the reproducible, residual inhibition displayed by the BMS-treated sample; perhaps some of the protease molecules did not return to the precise conformation they had prior to disulfide bonding, even after the disulfides were reduced. BMS had no effect on the activity of control CMV protease, consistent with lack of stimulation by DTT (Burck et al., 1994).

Identification of Cys138−Cys161 and Cys84−Cys87 Disulfide Pairs. To identify the disulfide pairs which form in HCMV protease, control and CL13933-treated A144L protease were alkylated and digested with trypsin. Half of the control and CL13933-treated digests were then treated with DTT, to reduce any disulfide bonds, and the resultant peptides were displayed by HPLC (Figure 4). For the control protease samples, the peptide profiles obtained with and without reduction by DTT are identical, due to the absence of disulfide linkages. All Cys-containing peptides in the control protease were alkylated, as demonstrated by earlier elution times from the C18 column compared to their free sulfhydryl counterparts (compare panels A and C). Several differences are apparent in comparing the nonreduced (panel B) and reduced (panel C) peptides from the CL13933-treated samples. A specific disulfide pair was detected linking Cys138 with Cys161 (at 62 and 64 min, panel B). Sequence analysis yielded two equimolar peptides presumably disulfide-linked at cysteines. When fully reduced to the free

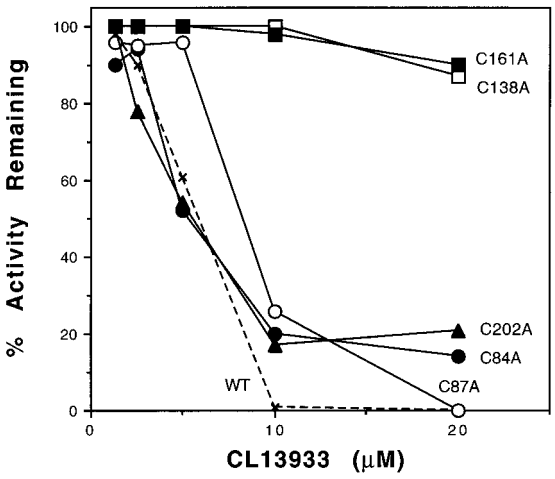


FIGURE 5: Inhibition of Cys mutants of HCMV protease by CL13933. Wild-type (WT) or Cys mutants of HCMV protease were incubated with the indicated concentrations of CL13933 in duplicate and assayed for activity by SPA. All proteases were used at ~3 μM, which gave ~75% cleavage of the peptide substrate in the absence of CL13933.

sulfhydryl peptides, new peaks eluting at 35.5, min, 36.5 (residues 157−165, residues 157−166), and 65 min (residues 137−156) were identified (panel C). Peptides containing Cys161 were detected in pairs due to incomplete tryptic digestion between Arg165 and Arg166. A second disulfide pair between Cys84 and Cys87 was identified by a sizable change in the elution time of peptide 80−92. Sequence analysis of this peptide yielded phenylthiohydantoincarboxyamidomethylcysteine from control protease (panel A), but phenylthiohydantoincystine (Hulmes et al., 1993) from CL13933-treated, nonreduced protease (panel B), confirming the Cys84−Cys87 disulfide. Peptides 202−215 and 202−232 were identified in the nonreduced map (panel B), but, upon reduction, these peptides disappeared and the reduced form of CL13933 was detected (panel C, 24 min), suggesting covalent attachment of one half CL13933 to Cys202. The covalent attachment of one half CL13933 to peptides 202−215 and 202−232, and the identities of all of the other Cys-containing peptides depicted in Figure 4, were confirmed by mass spectrometry (data not shown).

Thus, under these conditions, treatment of HCMV protease with CL13933 resulted in essentially complete disulfide bond formation between Cys138 and Cys161 and between Cys84 and Cys87. These data suggest that specific pairing of cysteines occurred, as opposed to scrambling or indiscriminate intermolecular disulfides. Evidence of an adduct between Cys202 and a half molecule of CL13933 was also detected.

Resistance of C138A, C161A Mutant Proteases to Inhibition by CL13933. A genetic approach was also taken to elucidate the role of particular cysteines of HCMV protease in inhibition of activity. Each of the five individual Cys residues was mutated to Ala, generating a panel of five mutant proteases (C84A, C87A, C138A, C161A, C202A). Each of the mutant proteases is enzymatically active, as judged by (i) autodigestion in *E. coli*, generating the 16- and 13-kDa cleavage products (data not shown), (ii) cleavage of authentic assembly protein in cotransfection studies (data not shown), and (iii) digestion of peptide substrate (Figure 5). The fact that these five mutant enzymes retain activity indicates that HCMV protease is not a Cys protease, consistent with previous data suggesting that herpesvirus proteases belong to the serine class (Welch et al., 1993; Holwerda et al., 1994; Liu & Roizman, 1992; DiIanni et al., 1994; Stevens et al., 1994).

The panel of mutant proteases was also tested for inhibition by CL13933 (Figure 5). Mutants C84A, C87A, and C202A are each inhibited by CL13933. The IC_{50} for mutants C84A, C87A, and C202A (~5 μM) is similar to that of wild type. In contrast, mutants C138A and C161A are not inhibited by CL13933. This observation, coupled with the finding that a substantial fraction (40%) of fully inhibited protease molecules contained only one disulfide (Figure 1G) and no CL13933 adduct (Figure 1C), strongly suggests that the disulfide bond between Cys138 and Cys161 can confer inhibition of the enzyme. An alternative explanation, that simultaneous attachment of compound to both Cys138 and Cys161 is required for inhibition, is unlikely since adduct formation is not necessary for CL13933 to inhibit wild type protease (Figure 1C).

Reactivity of Cys Residues Deduced from Loading of Excess Compound. To examine the reactivity of the five different Cys residues of HCMV protease, the panel of mutant Cys proteases was incubated with excess CL13933 and examined for covalent adduct formation by mass spectrometry (Figure 6A). Protease A144L, which is wild type with respect to cysteines, shows a major peak (67% of protease molecules) containing one half of CL13933, and a minor peak (33% of protease molecules) containing three halves of CL13933, consistent with previous data (Figure 1D). Focusing first on the major peaks, the mutants can be separated into three types, depending on the number of half-molecules of CL13933 which covalently attach to protease. The first type is C202A, in which the majority of protease molecules fail to attach any CL13933. Comparing the major peak of C202A protease with A144L protease (zero versus one half, Figure 6A) suggests that the major peak of A144L is due to loading of CL13933 on Cys202 and that removal of Cys202 (by mutation to Ala) abolishes this loading. These data are consistent with the peptide mapping studies (Figure 4) which indicated covalent attachment of CL13933 to peptides which contain Cys202.

The second type of loading, which is essentially identical to A144L, is demonstrated by C87A. The major peak of C87A, like A144L, is at one half CL13933. This suggests that the loading of CL13933 observed for A144L protease is not on Cys87, consistent with the loading being on Cys202, as discussed above.

The third type of loading, exhibited by proteases C138A, C161A, and C84A, is a major peak at two halves, compared to the one half seen for wild type A144L. Since the C138A, C161A, and C84A proteases still contain Cys202, then, presumably, residue Cys202 is responsible for loading one of the two half molecules of CL13933. The other, "extra" one half loading can be interpreted in conjunction with the disulfide pair data. We hypothesize that the extra one half loading of CL13933 on these mutants is on the now unpaired member of the disulfide pair. That is, for proteases C138A, C161A, and C84A, the loading of CL13933 is on Cys161, Cys138, and Cys87, respectively. The proposed loading sites for the major peaks for all of the proteases are presented in Figure 6B (lines marked "M").

For all of the proteases examined in the CL13933 loading experiments (Figure 6A), with the notable exception of the

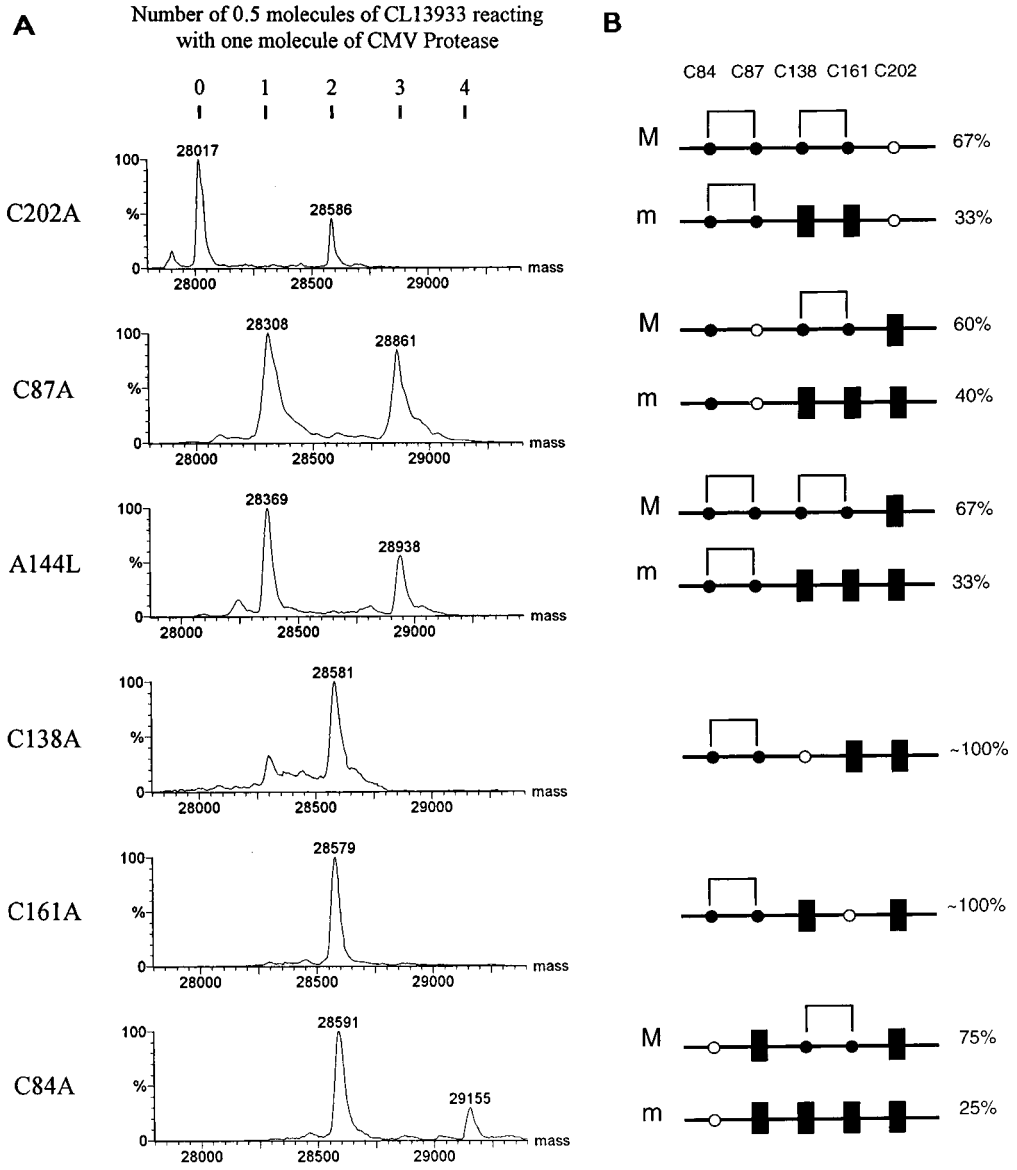


FIGURE 6: (A) Electrospray ionization mass spectra of Cys mutants of HCMV protease treated with CL13933. The indicated proteases were incubated with a 10-fold molar excess of CL13933 in 10 mM ammonium acetate, pH 7.5 (4 h at 25 °C). Protease A144L is wild type for cysteines; all other proteases contain a single Cys → Ala mutation (see text). (B) Proposed identity of the major (M) and minor (m) mass spectrometry peaks. HCMV protease is depicted as a line. Filled circles represent cysteines present in each protease; open circles are alanines substituted for cysteines. Filled rectangles are half molecules of CL13933. Disulfide bonds are indicated by bars connecting two cysteines.

C138A and C161A mutants, a minor peak which comprises 25−40% of the protease molecules is also observed. The identity of the residues which are loaded with CL13933 in this minor population can now be addressed. The model put forth in Figure 7 asserts that the thiols of Cys87, Cys138, and Cys161 can react with CL13933, forming a covalent adduct. At low concentrations of CL13933 (CL13933/protease < 10:1), the other member of the pair (Cys84, Cys161, and Cys138, respectively) forms a disulfide bond with its partner, by thiol−disulfide exchange. For the Cys84−Cys87 pair, it appears that the Cys84 residue itself does not attack CL13933, since removal of its partner in the C87A mutant protease did not result in "extra" loading onto Cys84, in contrast to mutants C84A, C138A, and C161A, which did exhibit "extra" loading onto the remaining partner (Figure 6A). Therefore, Cys84 is always available to displace compound from Cys87. However, in the Cys138−Cys161 pair, both partners are able to attack CL13933. At high concentrations of compound (CL13933/protease > 10:1), simultaneous addition of CL13933 to both residues Cys138 and Cys161 can occur, preventing displacement of compound and subsequent disulfide bond formation via thiol−disulfide exchange. Simultaneous attachment of CL13933 to residues Cys138 and Cys161 is postulated to be a "dead end" which is responsible for the minor peaks for CL13933 loading observed by mass spectrometry (Figure 6B, lines marked "m"). At high concentrations of CL13933, complete loading onto Cys202 is also postulated to occur, giving rise to two types of adduct: the major peak at one half and the minor peak at three halves of compound (Figure 7). The one half adduct is on Cys202 only; the three halves adduct are on Cys202, Cys138, and Cys161.

DISCUSSION

In this study, investigation of the mechanism of inhibition of HCMV protease by the compound CL13933 uncovered a previously unreported feature of the enzyme: the presence of multiple reactive cysteines which are capable of thiol−

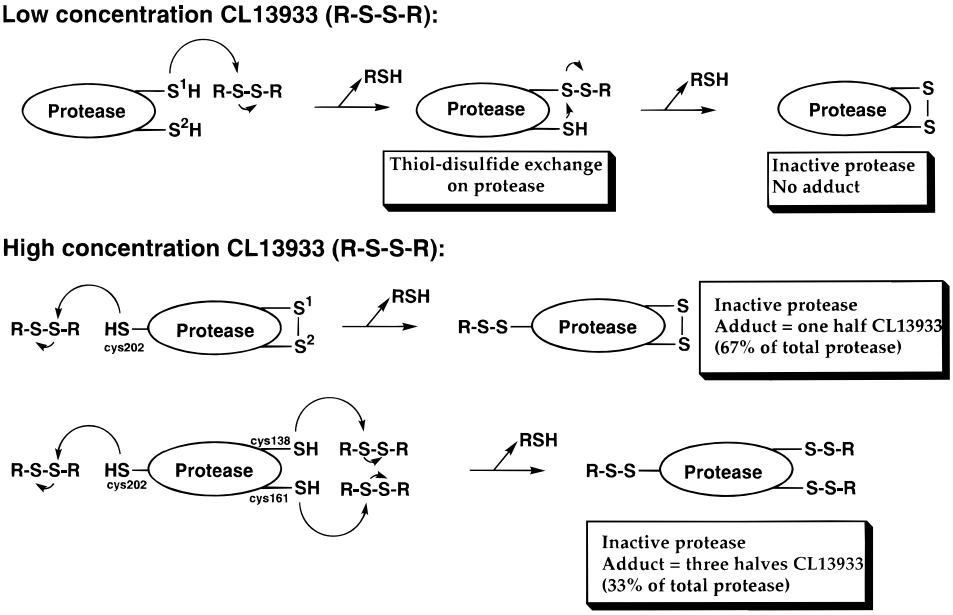


FIGURE 7: Model of reactions between CL13933 and cysteines of wild-type HCMV protease. At low concentrations of CL13933 (R−S−S−R) (experimentally defined as CL13933/protease < 10:1 for 2 h; Figure 1), only the most reactive of the Cys residues, Cys87, Cys138, and Cys161 (denoted by S^1), attack CL13933. When S^1 is Cys87, Cys138, or Cys161, then S^2 is Cys84, Cys161, or Cys138, respectively. The sulfhydryl S^2 performs thiol−disulfide exchange, expelling CL13933 and causing disulfide bond formation on HCMV protease. For simplicity, only one disulfide bond is shown, but two disulfides (Cys84−Cys87 and Cys138−Cys161) are formed. Disulfide Cys138−Cys161 apparently inhibits the protease. At high concentrations of CL13933, Cys202 is able to attach compound; this residue does not participate in disulfide bonding. Also at high concentrations of CL13933, Cys138 and Cys161 simultaneously make adducts with CL13933, preventing thiol−disulfide exchange between these residues.

disulfide exchange. The disulfide pairs are Cys84−Cys87 and Cys138−Cys161; Cys202 is unpaired. Protease mutants C138A and C161A are resistant to inhibition by CL13933, strongly suggesting that the Cys138-Cys161 disulfide in the wild-type protease is responsible for enzyme inhibition at low concentration of compound. Formation of this disulfide apparently occurs only via thiol−disulfide exchange and not by air oxidation, suggesting that Cys138 and Cys161 may not be in close proximity to each other until CL13933 induces a conformation change. Burck et al. (1994) also reported that promoting disulfide bond formation inhibits the protease, but the interactions between specific cysteine residues were not addressed.

At high concentration of CL13933, protease mutants C138A and C161A apparently load the compound on the remaining partner (Cys161 and Cys138, respectively; Figure 6) yet retain enzymatic activity. This suggests that a single adduct of CL13933 on either Cys138 or Cys161 can be tolerated. In contrast, simultaneous addition to both residues, which occurs in the wild-type protease containing three halves adduct of CL13933, inhibits the protease. This may be due to greater distortion of protease conformation from adducts at both Cys138 and Cys161, compared to an adduct at only one of these residues in the C138A and C161A mutant proteases. The size of the compound may be a factor: a larger compound may inhibit the mutant proteases.

Our results pose the question of whether the inhibition of HCMV protease by the Cys138−Cys161 disulfide observed *in vitro* actually occurs *in vivo* and potentially plays a role in regulating protease activity. We have noted that, upon expression in *E. coli* and transfected mammalian cells, the 85-kDa UL80 precursor is unstable and is cleaved rapidly into the 28-kDa protein. In contrast, during viral infection, the 85- and 80-kDa species exhibit a greatly reduced turnover rate (Welch et al., 1993; Jones et al., 1994). We previously postulated that regulated inhibition of protease activity occurs during viral infection and is not duplicated by the recombinant systems (Jones et al., 1994). It has been noted that Cys161 is conserved in all herpesvirus proteases but is dispensable for protease activity (Welch et al., 1993; DiIanni et al., 1994, and Figure 5). The lack of a direct catalytic function for Cys161, despite its conservation, argues for another role for this residue and is consistent with a potential role in protease regulation. It must be noted that Cys138 is not conserved and could not be the universal partner. Cys84 is conserved in the 28-kDa human, simian, and mouse CMV proteases (Welch et al., 1993; Loutsch et al., 1994); however, another Cys within the 85-kDa UL80 precursor (Cys645 is conserved), or in another protein (possibly virus-encoded) cannot be excluded.

The need to maintain viral proteases in an inactive state until the appropriate time in infection has been noted for other viruses. For HIV protease, activation occurs by dimerization (Krausslich, 1991), which is achieved by high concentrations of protease in the capsid, and recombinant HIV protease is a dimer *in vitro* (Meek et al., 1989). In contrast, HCMV protease is a monomer *in vitro* (Holwerda et al., 1994; Sardana et al., 1994).

Precedent exists for the regulation of viral protease by disulfide bond formation. The activity of adenovirus protease is dependent upon the formation of a disulfide-linked heterodimer between the protease and an 11 amino acid peptide cofactor derived from the C-terminus of the viral structural protein, pVI (Webster et al., 1993). Webster et al. (1993) have proposed that adenovirus protease is regulated via thiol−disulfide exchange and that heterodimer formation exposes the active site Cys. Although HCMV protease is a serine protease (Welch et al., 1993; Holwerda et al., 1994; Stevens et al., 1994) and a cofactor has not been identified, a mechanism similar to that of adenovirus protease could

occur. A disulfide involving conserved Cys161 could lock HCMV protease in an inactive conformation, which would be reversed by thiol−disulfide exchange. The activities of various cellular enzymes (Bauskin et al., 1991; Van Wart & Birkedal-Hansen, 1990; Ziegler, 1985) have been shown to be regulated by thiol−disulfide exchange, in response to the redox environment of the cell. It is possible that HCMV protease is redox-regulated: the UL80 precursor may be stable only in the cytoplasm of the infected cell and, upon transport to the nucleus and the developing viral capsid, undergo reduction, causing protease activation. In fact, recent data by Palamara et al. (1995) demonstrate that HSV-1 infection results in a decrease in intracellular glutathione levels which would render the cell more oxidizing, presumably promoting disulfide bond formation. In addition, treatment of infected cells with glutathione inhibited HSV-1 replication, by interfering with a late stage of virus replication (Palamara et al., 1995).

In summary, we have demonstrated that HCMV protease is capable of thiol−disulfide exchange, and that a specific intramolecular disulfide pair (Cys138−Cys161) results in inhibition of protease activity *in vitro*. It remains to be determined whether disulfide bond formation regulates the activity of the protease *in vivo*: the stability of the UL80 precursor may be due to its conformation, compartmentalization, or to another, unknown mechanism. Recombinant virus mutated at Cys161 would be useful in addressing this question.

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