Flavins Inhibit Human Cytomegalovirus UL80 Protease via Disulfide Bond Formation†

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ABSTRACT: Among the most potent inhibitors of human cytomegalovirus protease identified by random screening of a chemical library was 1,4-dihydro-7,8-dimethyl 6H-pyrimido[1,2-b]-1,2,4,5-tetrazin-6-one (**1**) (PTH_2). The oxidized form (**2**), PT, which is present in solutions of PTH_2, was shown to be the actual inhibitory species which irreversibly inactivates the protease; recycling of PTH_2 by dissolved oxygen results in complete inhibition of the protease at substoichiometric amounts of compound. No evidence for a covalent adduct between the protease and the inhibitor was obtained, and protease activity was restored by incubation of the inactivated enzyme with the reducing agent bismercaptoethyl sulfone, suggesting that disulfide bond formation was responsible for the observed inhibition. The five cysteines of the protease are normally in the reduced state; analysis of tryptic peptides from inhibited protease indicated that disulfide bonds Cys84−Cys87 and Cys138−Cys161 were formed. Using site-directed mutagenesis, the disulfide pair induced between Cys138 and Cys161 was shown to be essential for loss of enzymatic activity. Formation of the Cys138−Cys161 disulfide is dependent upon interaction of PT with the protease and does not form spontaneously, unlike that of the Cys84−Cys87 pair which can form in the absence of inhibitor. The inhibitor's redox chemistry is analogous to that of flavin, and, in fact, flavin inhibits the protease by the same mechanism, causing formation of a disulfide bond between Cys138 and Cys161. That the cysteines are dispensable, but can regulate protease activity by formation of a unique disulfide pair, suggests a plausible mechanism for control of proteolysis during the viral life cycle.

Human cytomegalovirus (HCMV), a betaherpesvirus, is an opportunistic pathogen of immunocompromised individuals, such as AIDS patients and recipients of organ or bone marrow transplants. The current treatments for HCMV disease, ganciclovir and foscarnet, inhibit the viral DNA polymerase but have limited effectiveness and display toxicity (Alford & Britt, 1993). Recently, the protease encoded by the HCMV UL80 gene has received attention as a possible target for antiviral chemotherapy, and details of its basic biology have begun to emerge (Welch et al., 1991; Baum et al., 1993; Jones et al., 1994; Burck et al., 1994). The 28-kDa HCMV protease (CMVP) is a serine enzyme (Welch et al., 1993; Stevens et al., 1994; Holwerda et al., 1994) which is autoproteolytically cleaved from an 85-kDa precursor. CMVP also cleaves the viral assembly protein precursor, an event necessary to encapsidate DNA into the virion (Preston et al., 1983, 1992; Rixon et al., 1988; Gao et al., 1994). CMVP contains five cysteines, each of which is dispensable for protease activity (Welch et al., 1993; Baum et al., 1996a). We have recently demonstrated that the topography of these cysteines make possible the formation of two specific intramolecular disulfide bridges, Cys84−Cys87 and Cys138−Cys161 (Baum et al., 1996a). Only the Cys138−Cys161 disulfide, which is formed by thiol/disulfide exchange, is associated with the loss of proteolytic activity.

In this study, we report on a new class of CMVP inhibitors that are structurally related to flavin. Inhibition was shown to proceed via an oxidation−reduction reaction involving the oxidized form of the inhibitor and sulfhydryl groups of CMVP. Two sets of disulfide pairs, Cys84−Cys87 and Cys138−Cys161, were identified; however, only the Cys138−Cys161 disulfide, which does not form spontaneously, results in the loss of enzymatic activity. Riboflavin and selected flavin analogs, but not nicotinamides, also inhibited CMVP. In many cellular oxidoreductases, flavins promote disulfide bond formation as part of the catalytic mechanism (Walsh, 1980; Stankovich, 1990). To the best of our knowledge, this is the first example of riboflavin and flavin analogs promoting intramolecular disulfide bond formation in a viral protein that result in the loss of enzymatic activity. The significance of this type of inhibition and its relationship to viral infection is discussed.

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

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[1] Abbreviations: HCMV, human cytomegalovirus; CMVP, cytomegalovirus protease; BMS, bismercaptoethyl sulfone; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-pressure liquid chromatography; HSV-1, Herpes simplex virus, type 1; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PT, 7,8-dimethyl-6H-pyrimido[1,2-b]-1,2,4,5-tetrazin-6-one; PTH_2, 1,4-dihydro-7,8-dimethyl-6H-pyrimido[1,2-b]-1,2,4,5-tetrazin-6-one.

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MATERIALS AND METHODS

Protease Constructs. All protease constructs are under T7 promoter control and are derivatives of plasmid p30k (Baum et al., 1993). Wild-type 28-kDa HCMV protease, which is capable of autodigestion into the 16- and 13-kDa proteins, and mutant A144L, which does not autodigest but is otherwise similar to wild type, have been described previously (Baum et al., 1996a). Mutant C138A/C161A was constructed by replacing the ~500 bp *Nde*I−*Eco*72I from p30k-C161A with the corresponding fragment from p30k-C138A (Baum et al., 1996a). Mutant C84S/C87S/C202A was constructed using the polymerase chain reaction with primer C84/87S-1 (5′ GGTCTTTTTTCCTTAGGCAGCGTCACTTCGCCC 3′) to introduce mutations C84S and C87S into p30k-C202A (Baum et al., 1996a). Proteases were purified as described (Baum et al., 1996a).

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., 1996b). 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 CMVP 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. Transformed 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, as described (Baum et al., 1996a). CMVP samples were desalted using Sephadex G-25 spin columns (Maniatis et al., 1982) and diluted to ~10 pM/μL in 3% acetic acid in 1:1 acetonitrile/water prior to injection. To determine the number of reduced cysteine residues, protease samples were denatured in 6 M urea/50 mM Tris-Cl, pH 7.5 (20 min at 22 °C) and treated with the alkylating agent iodoacetamide (15 mM, 15 min at 22 °C) (Means & Feeney, 1971). After spin column chromatography, protease samples were then subjected to mass spectrometry to determine the number of alkyl (carboxyamidomethyl) groups covalently attached to protease, from the mass shift of 57 Da per group.

HPLC of PTH2/PT. For the experiment shown in Figure 3, PT (44 μM) was incubated with CMVP (11 μM) in 1 mL of 50 mM Tris-Cl, pH 7.5/50 mM NaCl at 20 °C. At the indicated times, aliquots (200 μl) were injected and chromatographed on a Vydac C18 reverse-phase column (250 × 4.6 mm) to monitor the formation of PTH_2 and the disappearance of PT. The gradient HPLC system was as follows: flow rate, 1 mL/min; mobile phase A, 0.1% TFA; mobile phase B, CH_3CN; gradient, 8−25% B in 10 min; detection at 275 nm.

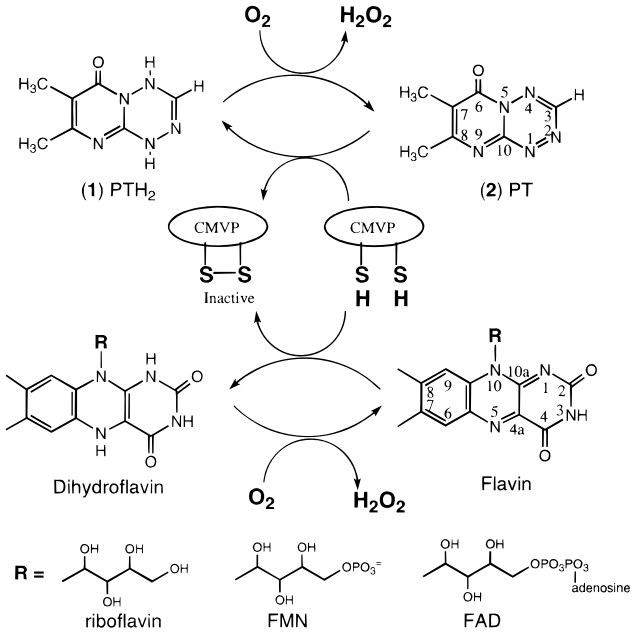

FIGURE 1: Redox recycling of PT and flavins and disulfide bond formation on CMVP. PTH_2 oxidizes to PT, presumably due to dissolved oxygen (from air) present in the solution; PT causes oxidation (disulfide bond formation) of CMVP and undergoes concomitant reduction to PTH_2. For clarity, only the inhibitory disulfide between Cys138 and Cys161 is shown; a second disulfide bond is formed between Cys84 and Cys87. The analogous redox reaction of riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) is shown. Production of H_2O_2 occurs upon oxidation of dihydroflavin (Walsh, 1980) and is assumed to occur upon oxidation of PTH_2.

Cyclic Voltammetry. Voltammetric measurements were conducted with a Bioanalytical System Inc. (BAS)-100 electrochemical apparatus interfaced with a digital plotter (BAS-PL100). The electrochemical cell consisted of a three electrode system: a glassy carbon working electrode (3.2 mm diameter, BAS-GCE), Ag/AgCl/(3 M) aqueous NaCl reference electrode, and a platinum wire auxiliary electrode. Prior to each electrochemical measurement, the sample was degassed with argon.

RESULTS

Identification of PT as a Potent Inhibitor of CMVP. Through random screening of a chemical library, the 179-Da compound PTH_2 (**1**) (1,4-dihydro-7,8-dimethyl 6H-pyrimido[1,2-b]-1,2,4,5-tetrazin-6-one; Bitha et al., 1987) was identified as a potent inhibitor of CMVP ($IC_{50} \sim 1$ μM). However, in the absence of CMVP, PTH_2 was converted to a new species upon dissolving in buffer (50 mM Tris-Cl, pH 7.5, at 20 °C), as demonstrated by a change in retention time on HPLC. Electrospray mass spectrometry and NMR indicated that PTH_2 (dihydro form) is readily oxidized ($t_{1/2}$ ~27 min) under these experimental conditions to yield PT (177 Da, **2**). This reaction is depicted at the top of Figure 1; the oxidant is apparently dissolved oxygen (from air) present in the solution. Evidence that the oxidized form, PT, and not PTH_2, is the actual species responsible for the inhibition of CMVP is provided by the following observations. PTH_2 (35 μM) failed to inhibit CMVP in the presence of the reducing agents dithiothreitol (1 mM) or ascorbic acid (6 mM). Also, the 1,4-dimethyl derivative of PTH_2, which cannot undergo oxidation, failed to inhibit CMVP. Finally,

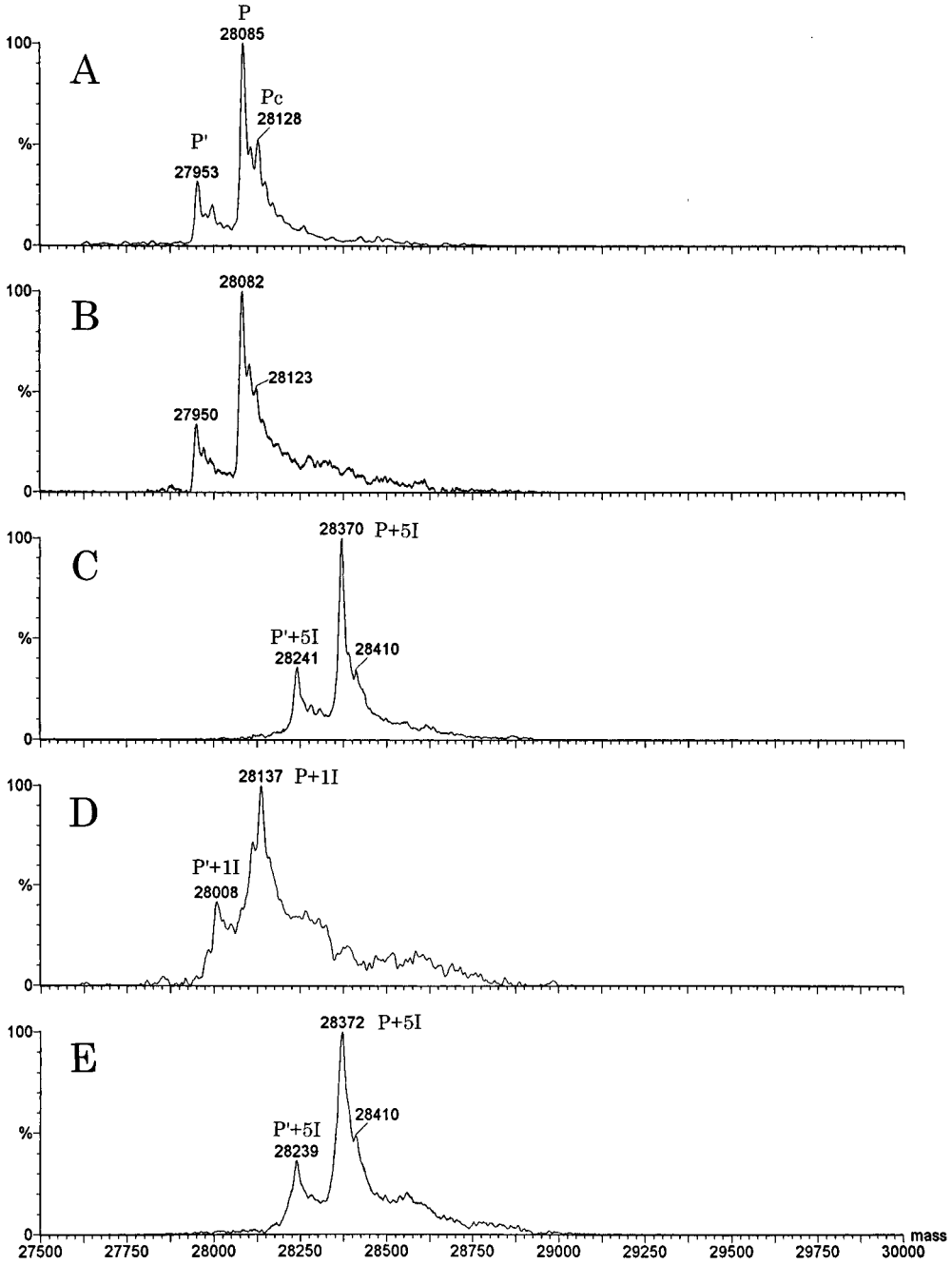

FIGURE 2: Electrospray ionization mass spectrometry of CMVP. A144L protease (75 μM) was incubated without (panel A) or with PT (600 μM) (panel B) in 0.1 mL of 10 mM Tris-Cl, pH 7.5/50 mM NaCl for 2 h at 25 °C. Samples were desalted and PT removed by G-25 spin column chromatography. Samples (10 μL) were then analyzed by mass spectrometry. For alkylation studies (panels C−E), aliquots of the chromatographed control and PT-treated samples were denatured in 6 M urea and incubated with IAM as described in Materials and Methods, followed by spin column chromatography and mass spectrometry. (Panel C) No PT. (Panel D) PT treated. (Panel E) PT treated, with 7.5 mM BMS present during denaturation. The protease (P) peaks and the number of alkyl groups (I) are shown. P′ lacks the amino-terminal methionine residue and behaves identically to its methionine-containing counterpart; Pc results from carbamylation of protease during resuspension of *E. coli* inclusion bodies in urea (Baum et al., 1996a).

incubation of the protease with HPLC-purified PT resulted in inhibition of CMVP.

Inhibition of CMVP by PT was explored further in a series of experiments designed to determine the mechanism of protease inhibition. Inhibition of CMVP by this compound was found to be irreversible, as defined by the failure to recover active enzyme upon removal of the compound by dialysis or by gel filtration chromatography using Sephadex G-25. The G-25 eluate of CMVP incubated with PT was analyzed by electrospray mass spectroscopy for the protein and the compound. The mass of the PT-treated protease was identical (within experimental error) to that of the untreated protease (Figure 2, panels A and B), and no PT was detected in the G-25 eluate. Thus, the irreversible nature of the inhibition is not due to formation of a stable covalent adduct between CMVP and PT, and no evidence of a tight noncovalent association of the compound with the enzyme was observed.

Recycling of PTH_2 by Air Oxidation. Evidence that inhibition of CMVP could be due to oxidation of a functional group(s) on the protease was suggested by the observation that incubation of PT (**2**) with the protease resulted in its conversion to the dihydro form, PTH_2 (**1**). PT was mixed with CMVP (molar ratio PT/CMVP of 4:1), and the amount

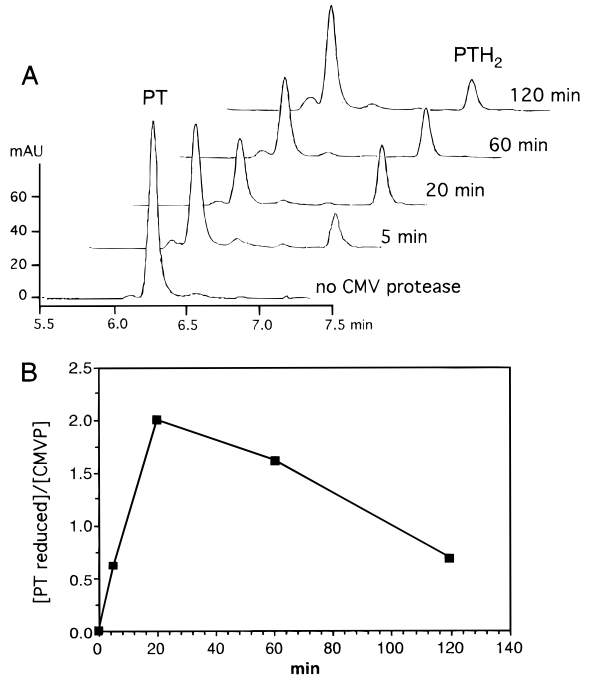

FIGURE 3: (A) Cycling of PT and PTH_2 in the presence of CMVP as a function of time. PT (44 μM) was incubated with CMVP (11 μM) for 5, 20, 60, or 120 min, in 50 mM Tris-Cl, pH 7.0/50 mM NaCl, and analyzed by HPLC as described in Materials and Methods. Peaks corresponding to PT and PTH_2 are indicated. A control sample of PT incubated without CMVP remained entirely as PT during the course of the incubation; only the 5 min sample is shown. (B) Time course of reduction of PT in the presence of CMVP. The amount of PT reduced to PTH_2 was calculated from the data shown in panel A as follows: $[(A_0 - A_t)/A_0] \times [44\ \mu M\ PT/11\ \mu M\ CMVP]$, where A_0 is the peak area of PT without CMVP; A_t is the peak area of PT incubated with CMVP at a specified time.

of oxidized and reduced species at various times of incubation was determined by HPLC (Figure 3). Reduction of PT does not occur spontaneously or when the protease was replaced with bovine serum albumin (data not shown) but is dependent upon the presence of CMVP (Figure 3A). After 20 min, ~50% of PT was reduced to PTH_2. Since PTH_2 is rapidly converted to PT, apparently by dissolved oxygen in the buffer as noted above, the PTH_2 formed is oxidized to regenerate PT, causing a fluctuation in the amount of PTH_2 detected (Figure 3A). Figure 3B shows the amount of PT reduced to PTH_2 during the course of the reaction in terms of molar equivalents of CMVP; at the maximum, approximately 2 mol of PT is reduced to PTH_2 per mole of protease.

PT exhibited an IC_{50} of 0.3 μM in the CMVP activity assay, using a 2 h preincubation of CMVP with PT prior to the addition of peptide substrate (Table 1). CMVP is itself present in the assay at 2 μM; the requirement of relatively high protease concentration for substrate cleavage is typical of herpesvirus proteases (Baum et al., 1993; Burck et al., 1994; Sardana et al., 1994; Stevens et al., 1994). Doubling the CMVP concentration in the assay resulted in a doubling of the observed IC_{50}, for the same 2 h preincubation period of CMVP and PT. The apparent substoichometric inhibition of CMVP by PT observed under these conditions is consistent with redox recycling of the inhibitor (Figure 1). The IC_{50} increased with shorter preincubation times; without preincubation, the IC_{50} increased to 2 μM, becoming stoichiometric in the absence of time for recycling to occur.

Table 1: Inhibition of HCMV Protease by Flavins and by GSSG[a]

compound	IC_{50} μM
PT	0.3
riboflavin	0.3
flavin adenine dinucleotide	≫40
flavin mononucleotide	0.3
nicotinamide	≫100
nicotinamide adenine dinucleotide	≫20
nicotinamide adenine dinucleotide phosphate	≫20
GSH	≫10 000
GSSG	300

[a] Protease activity was determined by scintillation proximity assay as described in Materials and Methods. Wild-type protease was used at 2 μM. Flavins and nicotinamides (oxidized forms), GSH, and GSSG were purchased from Sigma. For PT, riboflavin, FMN, and GSSG, the IC_{50} is shown; the other compounds did not inhibit CMVP at the highest concentration tested (e.g., 10 mM GSH).

PT Causes Formation of Internal Disulfide Bonds on CMVP. The reduction of PT in the presence of CMVP suggests that CMVP must be oxidized during this process. The most likely protein side chain candidates are those of the five cysteine residues of CMVP (Cys84, 87, 138, 161, and 202) which could conceivably be oxidized to disulfides. Inactivation of CMVP via disulfide bond formation is indeed indicated, since the enzymatic activity of the protease inhibited by PT is restored upon treatment with the reducing agent bismercaptoethyl sulfone (BMS, 10 mM). Intramolecular, rather than intermolecular, disulfide bond formation is likely to result from PT treatment of CMVP, since no evidence for the formation of dimeric or higher order structures was obtained by mass spectrometry (Figure 2B) or by nonreducing SDS polyacrylamide gel electrophoresis (data not shown). Also, such a mechanism of inactivation would explain the lack of a significant mass difference between control and PT-treated CMVP, since the loss of 2 or 4 Da from the 28 085 Da CMVP cannot be accurately detected by electrospray mass spectrometry.

To determine the oxidation status of the five cysteines of CMVP, iodoacetamide was used as a chemical probe. This reagent alkylates sulfhydryl groups of cysteines not engaged in disulfide bonds (Means & Feeney, 1971). CMVP was incubated in the presence and absence of PT, subjected to G-25 chromatography (to remove PT), denatured in 6 M urea, and alkylated using iodoacetamide. The number of sulfhydryl groups was determined by electrospray mass spectrometry of the protein, calculating the number of multiples of 57 Da [the mass of the alkyl (carboxyamidomethyl) group] which added to the mass of CMVP. Control protease has five free cysteines (Figure 2C), in agreement with a previous report that CMVP does not normally contain disulfides (Burck et al., 1994; Pinko et al., 1995). In contrast to the untreated protein, PT-treated CMVP added only one alkyl group per protein molecule (Figure 2D), indicating that two disulfide bonds are formed on CMVP as a result of incubation with PT. This result is consistent with the reaction stoichiometry presented in Figure 3B, showing that 2 mol of PT is reduced per mole of CMVP.

To unambiguously ascribe the differences in the alkylation patterns for control and PT-treated CMVP to disulfide bond formation, PT-treated enzyme was treated with the reducing agent BMS prior to alkylation. As mentioned above, this treatment restored protease activity. BMS is superior to dithiothreitol (DTT) at reducing disulfide bonds at neutral

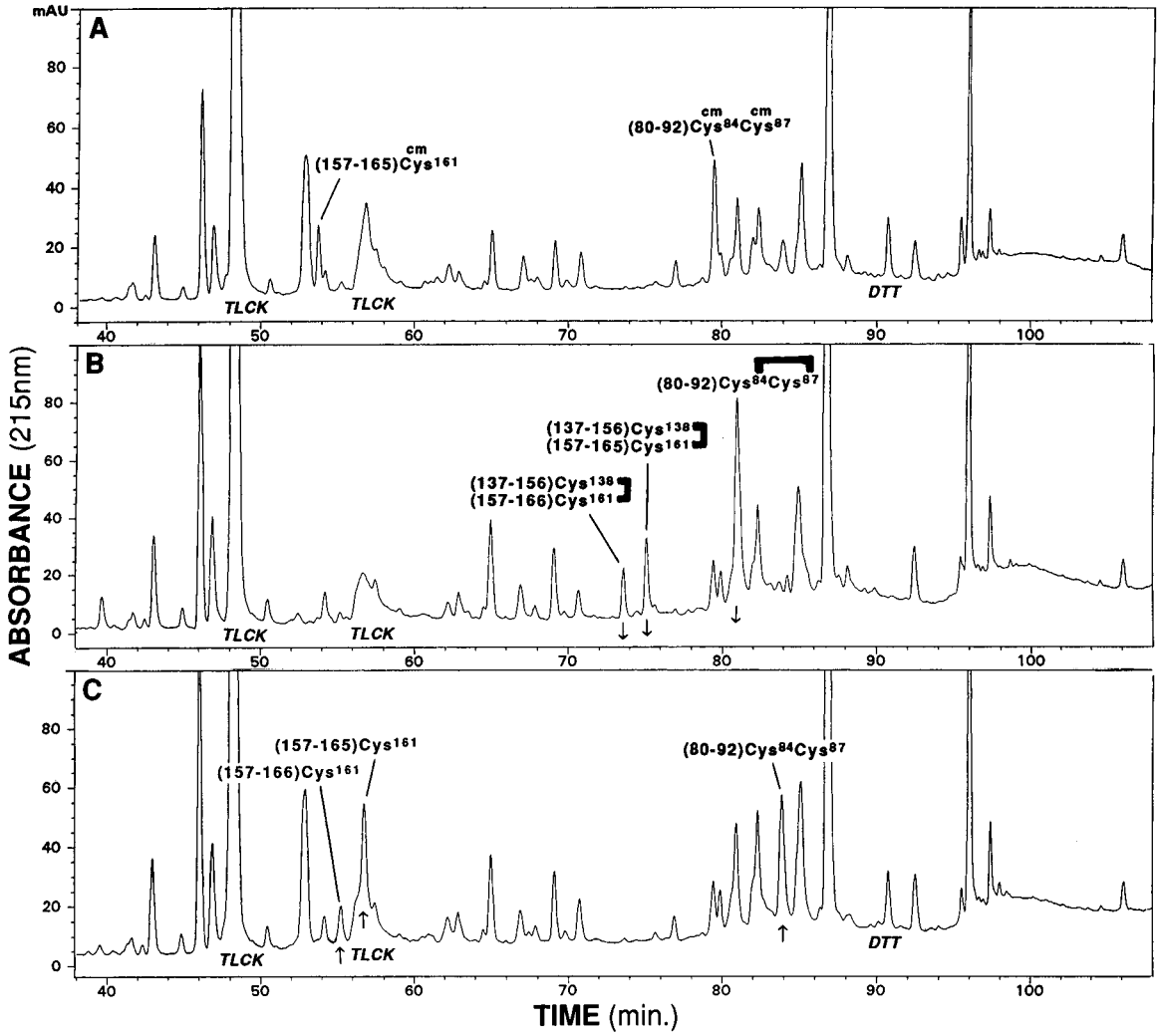

FIGURE 4: Separation of tryptic peptides of control and PT-treated CMVP, and identification of disulfide pairs Cys138−Cys161 and Cys84−Cys87. (A) Tryptic peptides from control, carboxyamidomethylated A144L CMVP were subjected to reverse-phase HPLC and eluted with a gradient of acetonitrile (0% for 10 min, to 32% in 70 min, to 80% in 20 min at 100 μL/min) in 0.05% trifluoroacetic acid. The DTT-reduced map (shown) is identical to the nonreduced map. Numbers in parentheses refer to the first and last amino acid residues of a given peptide; Cys^{cm} denotes carboxyamidomethylcysteine. (B) Tryptic peptides from PT-treated, carboxyamidomethylated CMVP. Disulfide-linked peptides are shown by bars connecting Cys residues. (C) Tryptic peptides from PT-treated, carboxyamidomethylated CMVP, following treatment with DTT. Peaks introduced by DTT or the trypsin inhibitor TLCK are indicated. Arrows denote the disappearance (↓) and appearance (↑) of peptides upon DTT treatment.

pH (Singh, & Whitesides, 1994); neither DTT nor reduced glutathione (GSH) (at 10 mM) restored protease activity. The pK_a's of BMS, GSH, and DTT are 7.8, 8.7, and 9.2, respectively (Singh & Whitesides, 1994; Lees & Whitesides, 1993); therefore, in our experiments at pH 7.5, only BMS has a significant fraction of the reactive thiolate anion present, which facilitates reduction of protein disulfides. As shown in Figure 2E, the PT-treated sample, upon reduction with BMS, now shows addition of five alkyl groups, demonstrating five free cysteine residues. Thus, interaction of PT with CMVP induces formation of two internal disulfide bonds that result in the loss of enzymatic activity.

Disulfide Pairs Cys138−161 and Cys84−87 Are Formed by PT Treatment of CMVP. CMVP contains five cysteine residues, at positions 84, 87, 138, 161, and 202. To identify which cysteine residues are paired upon incubation with PT, control and PT-treated CMVP were each alkylated with iodoacetamide to prevent any thiol−disulfide exchange and digested with trypsin (Baum et al., 1996a). One half of each tryptic digest was reduced with DTT, and the resulting peptides were displayed by HPLC and compared to the unreduced digest (Figure 4). Peptides which exhibited altered retention times were then subjected to N-terminal sequence analysis. For the control CMVP, the reduced and non-reduced maps are identical, consistent with the absence of disulfide bonds; only the reduced map is shown (panel A). Several differences are apparent in comparing the reduced and nonreduced maps of PT-treated CMVP (panels B and C, arrows). In the nonreduced map, a specific disulfide pair was detected linking Cys138 with Cys161 (at 74 and 75 min, panel B). Sequence analysis yielded equimolar amounts of two peptides (aa137−aa156 and aa157−aa165) presumably disulfide-linked at cysteines. Upon reduction with DTT, new peaks containing Cys161 at 55 and 57 min were detected (residues 157−165 and residues 157−166, respectively); peptides containing Cys161 are detected in pairs due to incomplete tryptic digestion between Arg165 and Arg166. Peptide which contains Cys138 was not detected upon reduction (panel C) or in the control CMVP map (panel A); this peptide may not be soluble. A second disulfide pair between Cys84 and Cys87 was identified by a sizable change in the elution time of peptide 80−

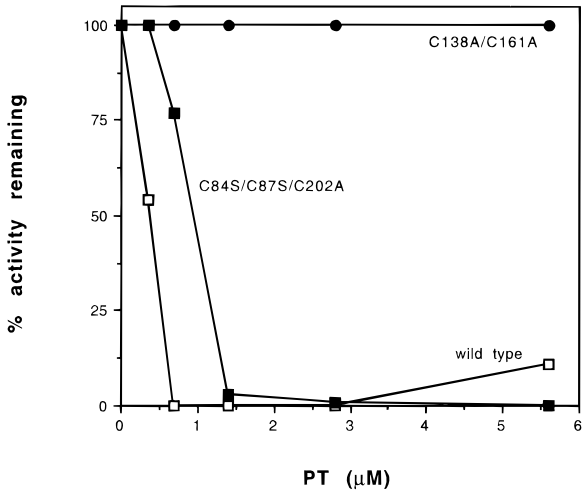

FIGURE 5: Resistance of C138A/C161A mutant CMVP, and sensitivity of C84S/C87S/C202A mutant CMVP, to inhibition by PT. Protease activity was monitored by cleavage of radiolabeled peptide substrate by scintillation proximity assay as described in Materials and Methods. Wild-type and C84S/C87S/C202A proteases were ~2 μM, and C138A/C161A protease was ~4 μM; these concentrations result in ~75% cleavage of the peptide substrate in the absence of PT, as described in the text. The experiment shown represents two independent determinations.

92. These data indicate that PT treatment of CMVP results in disulfides between Cys84 and Cys87 and between Cys138 and Cys161. In contrast, Cys202 did not participate in disulfide bonding.

Identification of Cys138−Cys161 as the "Inhibitory" Disulfide Pair. Since two disulfide pairs are formed on CMVP upon treatment with PT, we sought to establish if one or both disulfide pairs are required for inhibition of protease activity. Using site-directed mutagenesis, mutant proteases C138A/C161A (which can still form the Cys84−Cys87 disulfide) and C84S/C87S/C202A (which can still form the Cys138−Cys161 disulfide) were constructed to separately analyze the contribution of the Cys84−Cys87 and Cys138−Cys161 disulfide pairs to protease inhibition. The mutant proteases retained enzymatic activity, as demonstrated by autodigestion of the 28-kDa mutant proteases into the 16- and 13-kDa fragments (as detected by Coomassie staining of proteases subjected to SDS−polyacrylamide gel electrophoresis, data not shown) and also by cleavage of peptide substrate in the SPA. For the SPA experiments, all proteases were first titrated (without potential inhibitors), to determine the minimal amount required to achieve ~75% cleavage of substrate. For wild type and C84S/C87S/C202A, ~2 μM protease was required; for C138A/C161A, ~4 μM protease was required, indicating that both the C138A/C161A and C84S/C87S/C202A mutant proteases are enzymatically active. Previously, we and others have shown that each individual cysteine can be replaced by alanine with retention of protease activity (Baum et al., 1996a; Welch et al., 1993); the current data extend this finding to simultaneous replacement of multiple cysteines.

Wild-type CMVP and mutants C138A/C161A and C84S/C87S/C202A were examined for inhibition by PT (Figure 5). In this experiment, PT exhibited an IC_{50} of about 0.4 μM for wild-type CMVP. Mutant C84S/C87S/C202A CMVP was also inhibited by PT, with IC_{50} ~1 μM, similar to wild-type protease. In contrast, mutant C138A/C161A CMVP was resistant to inhibition by PT. Increasing the PT concentration to 50 μM still failed to inhibit C138A/C161A CMVP (Figure 5 and data not shown). These data strongly suggest that formation of the Cys138−Cys161 disulfide pair, which can form in the CMVP mutant C84S/C87S/C202A, but not in the CMVP mutant C138A/C161A, confers inhibition of protease activity. In contrast, formation of the Cys84−Cys87 disulfide pair, which can form in the CMVP mutant C138A/C161A, but not in the CMVP mutant C84S/C87S/C202A, does not appreciably inhibit the enzyme. Additional support for the involvement of the Cys138−Cys161 disulfide pair in inhibition of protease activity comes from a previous study of the five individual cysteine mutants of CMVP; mutant proteases C138A and C161A were each resistant to inhibition by the disulfide compound CL13933 [1,1′-(dithio-di-*o*-phenylene)-*bis*-(5-phenylbiguanide)], which facilitates intramolecular disulfide bond formation via protein thiol−mixed disulfide exchange (Baum et al., 1996a).

The two mutant enzymes, C84S/C87S/C202A and C138A/C161A, also allowed us to separately examine the ability of disulfides Cys84−Cys87 and Cys138−Cys161 to form spontaneously by dissolved oxygen, in the absence of the inhibitor PT. The mutant proteases were incubated (3 h at 22 °C in 10 mM Tris-Cl, pH 7.5) and then subjected to carboxyamidomethylation under the same conditions as Figure 2 (data not shown). For protease C138A/C161A, 55% of the protease molecules added three alkyl groups and 45% added only one alkyl group, indicating that 45% of the protease molecules formed disulfide Cys84−Cys87 by air oxidation. The activity of this protease is not stimulated by reducing agent, adding further support that the Cys84−Cys87 disulfide does not impair enzymatic activity. In contrast, alkylation of protease C84S/C87S/C202A gave 100% with two alkyl groups per CMVP molecule, indicating that all cysteines were in the reduced form and that the Cys138−Cys161 disulfide does not form by air oxidation. That the Cys138−Cys161 disulfide does not form spontaneously suggests that the binding of PT to CMVP may induce a conformation change in the protease, permitting Cys138 and Cys161 to come into sufficiently close proximity to allow disulfide bond formation.

Similarity of PT to Flavins. The redox chemistry exhibited by PTH_2/PT is reminiscent of flavin (Figure 1). Therefore, several flavin analogs were tested for the ability to inhibit CMVP (Table 1). Riboflavin and flavin mononucleotide (FMN) inhibited CMVP with IC_{50}'s of about 0.3 μM and are as potent as PT (Table 1), in assays using 2 μM protease. The substoichiometric inhibition of CMVP by these flavins, like PT, may be explained by recycling of the reduced flavin by dissolved oxygen in the solution. Inhibition was not detected if the reducing agent BMS (2 mM) was present.

The ability of riboflavin to induce disulfide bond formation in CMVP was examined. CMVP and riboflavin-treated CMVP were analyzed by electrospray mass spectrometry as described in Figure 2 (data not shown). Riboflavin did not form a stable covalent adduct with CMVP. As expected, alkylation of untreated CMVP showed addition of five alkyl groups, whereas the riboflavin-treated enzyme showed evidence of substantial disulfide bond formation. About 35% of the protease molecules contained two disulfides, and 40% contained one disulfide; only 25% of the protease molecules contained no disulfides (data not shown). In accord with the studies on PT, riboflavin failed to inhibit the mutant protease C138A/C161A. Together, these data show that

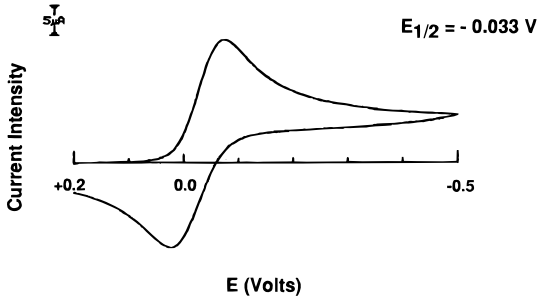

FIGURE 6: Cyclic voltammogram of PT (0.1 mM in phosphate buffered saline, pH 7.2) at the glassy carbon electrode. The conditions were working/auxiliary/reference electrodes: glassy carbon/platinum/Ag-AgCl (3 M aqueous NaCl) and a scan rate of 100 mV/s. The $E_{1/2}$ of −0.033 V and the potential axis are defined relative to the Ag/AgCl electrode. The electrochemical experiments were conducted under anaerobic conditions, in the presence of argon.

riboflavin and PT inhibit CMVP by the same mechanism, by promoting disulfide bond formation between Cys138 and Cys161.

In contrast to riboflavin and FMN, flavin adenine dinucleotide (FAD) did not inhibit CMVP (Table 1). FAD is significantly larger than either riboflavin or FMN (Figure 1) and may be unable to gain access to the cysteines of CMVP. Nicotinamides, like flavins, participate in biological redox chemistry but exhibit several differences: (i) nicotinamides are restricted to two-electron transfer, whereas flavins can participate in one- and two-electron transfers, and (ii) dihydronicotinamide cannot be oxidized by oxygen in the absence of a catalyst, even though the redox activity is thermodynamically favored. In contrast, dihydroflavin does react with oxygen (Walsh, 1980). It was of interest to determine whether nicotinamides inhibit CMVP. Nicotinamide, NAD, and NADP were tested and do not inhibit CMVP (Table 1). The lack of inhibition by the nicotinamides could result from unfavorable redox potentials (E_0 = −0.32 V; Lees & Whitesides, 1993) and from structural differences between the nicotinamide ring and the ring systems of PT (tetrazine) and riboflavin (isoalloxazine).

The reduction potentials of PT and riboflavin were determined by cyclic voltammetry to gain insight into the chemical mechanism of the redox inhibition of CMVP by these compounds. The cyclic voltammogram of PT (Figure 6) showed a cathodic peak at −0.07 V and an anodic peak at 0.004 V, and the $E_{1/2}$ value (relative to the Ag/AgCl electrode) was calculated to be −0.033 V. No new peaks were observed during multicycle scanning or when the scan was reversed in the oxidation direction. These results are consistent with a two-electron, reversible reduction of PT and formation of electrochemically stable PTH_2 (argon atmosphere, 100 mV/s scan rate). The observed reduction potential of −0.033 V for PT corresponds to an E_0 of 0.2 V on the normal hydrogen electrode scale (using the E_0 of 0.23 V for the Ag/AgCl electrode). The corresponding E_0 value determined for riboflavin under these conditions is −0.19 V (data not shown), indicating that PT is the more potent oxidizing agent. For disulfide bond formation to occur, the E_0 of the oxidant should be more positive than that of the cysteines of interest; although data for CMVP are unavailable, the E_0 for various individual protein disulfides is ∼ −0.2 to −0.4 V (Holmgren, 1981, and references therein). Therefore, reduction of PT and riboflavin, and concomittant disulfide bond formation between cysteine residues, is thermodynamically favorable.

Inhibition of CMVP by GSSG. Glutathione (γGluCysHGly), the major intracellular redox buffer, exists in reduced and oxidized forms (GSH and GSSG, respectively) and is present at millimolar concentration in most cell types [reviewed in Gilbert (1995) and Meister (1995)]. It was of interest to test the effects of GSH and GSSG on CMVP activity. As shown Table 1, GSH (10 mM) had no effect on CMVP activity, consistent with the behavior of other reducing agents such as DTT or BMS (Burck et al., 1994; Baum et al., 1996a). In contrast, GSSG inhibited CMVP *in vitro* in the SPA (IC_{50} ∼300 μM, Table 1). Mass spectroscopic analysis of CMVP incubated with GSSG (E. Baum, G. Bebernitz, K. Tabei, and M. Siegel, unpublished observations) indicated that mixed disulfides between glutathione and CMVP were formed. We assume that the formation of covalent adducts on CMVP by glutathione is analogous to that observed for the CMVP inhibitor CL13933 (Baum et al., 1996a). Like GSSG, CL13933 is a symmetrical disulfide compound, and half molecules of CL13933 become covalently attached to Cys residues of CMVP (Baum et al., 1996a). The formation of mixed protein−glutathione disulfides has been documented for several other enzymes, including glycogen phosphorylase, phosphofructokinase, and hydroxymethylglutaryl-CoA reductase (Gilbert, 1995, and references therein).

DISCUSSION

We report here that flavin and a flavin-like molecule, PT, irreversibly inhibit HCMV protease by promoting intramolecular disulfide bond formation. Loss of proteolytic activity was ascribed to the formation of a disulfide pair between Cys138 and Cys161 that does not occur readily by air oxidation but is specifically induced by the interaction of the inhibitors with the protease. A second disulfide pair, Cys84−Cys87, is established by the inhibitor−protease interaction; however, this pair can also form by air oxidation and does not impair the enzymatic activity of the protease.

The ability of flavins to induce disulfide bond formation in a number of cellular oxidoreductases, such as dihydrolipoamide dehydrogenase, glutatathione reductase, and thioredoxin reductase, is well-documented (Walsh, 1980; Stankovich, 1990). To our knowledge, flavins induce disulfides only in the limited group of enzymes for which they serve as cofactors and are not general reagents for promoting protein disulfide bond formation; thus, the ability of flavin to induce disulfides on CMVP is noteworthy. Model studies propose that the mechanism for the oxidation of dithiols to disulfides by oxidized flavins proceeds via addition of a thiol to the C4a of the flavin. Attack at sulfur by a second thiolate anion in close proximity to the C-4a flavin−thiol adduct results in disulfide formation and transfer of electrons to the flavin. For a flavoenzyme, the second half of the reaction proceeds with the catalytic reoxidation of the flavin facilited by a nicotinamide cofactor. For CMVP, the dihydroflavin appears to be reoxidized by dissolved oxygen upon release into solution. Flavin-containing enzymes that form intramolecular disulfides as part of their normal catalytic mechanism are specialized cases where two cysteines are in close proximity to each other at the active center. In CMVP, two disulfides are formed at the expense of the reduction of two

molecules of flavin, and, by analogy to flavin, we propose that either Cys138 or Cys161 attacks position 10 of PT (Figure 1), facilitating disulfide bond formation.

A convergence of data from two ostensibly dissimilar compounds, the disulfide CL13933 (Baum et al., 1996a) and the redox active molecule PT, is evident. Both molecules are thiophiles and exert inhibition at the level of disulfide bond formation between Cys138 and Cys161. A variety of alkylating and acylating agents that inhibit CMVP apparently also target these residues, forming a covalent adduct on either Cys138 or Cys161 which is stable and cannot be expelled by thiol−disulfide exchange. For example, phenylmethanesulfonyl fluoride, which we previously reported inhibits CMVP (Baum et al., 1993), forms multiple covalent adducts with the protease as seen by mass spectrometry, and the C138A/C161A mutant protease is resistant to inhibition by this compound (data not shown), suggesting that inhibition of the wild-type enzyme was via cysteine residues and not via the active site Ser132. Considerable evidence indicates that CMVP is a serine protease (Welch et al., 1993; Holwerda et al., 1994; DiIanni et al., 1994; Stevens et al., 1994), but it is resistant to several general serine protease inhibitors and lacks homology to known serine proteases, suggesting that it may represent a new serine protease family (Welch et al., 1993; DiIanni et al., 1994).

It is possible that the Cys138−Cys161 disulfide is close to, or at, the active site of the enzyme. The active site nucleophile of CMVP, Ser132 (Welch et al., 1993; Stevens et al., 1994; Holwerda et al., 1994) is close to Cys138 from the primary sequence. Since Cys138 and Cys161 are close enough to form a disulfide in the presence of flavin or PT, Ser132 may also be near Cys161 in three-dimensional space. Alternatively, these cysteine residues could be remote from the active site but stabilize (via chemical modification or by disulfide bond formation) a conformation of the enzyme that is catalytically impaired by distortion or misalignment of catalytic and binding groups at the active center. In fact, preliminary circular dichroism studies of control and PT-treated CMVP suggest that a conformation change does indeed occur upon disulfide bond formation, as evidenced by a small but significant increase (∼10%) in dichroic absorption at 206 nm (G. Krishnamurthy, unpublished observations).

The presence of multiple reactive cysteines on CMVP is reminiscent of ribonucleotide reductase which catalyzes the conversion of nucleotides to deoxyribonucleotides and is found in all eukaryotes, several viruses, and some prokaryotes (Mao et al., 1989, 1992; Sjoberg, 1994). Ribonucleotide reductase is composed of two each of the R1 and R2 subunits; R1 contains the five reactive cysteines, and R2 contributes a tyrosyl radical. Four cysteines, in close proximity, are oxidized to two disulfide pairs upon reduction of the substrate and can be reduced by thioredoxin, thioredoxin reductase, NADPH, or dithiothreitol, to regenerate the enzyme. Interestingly, ribonucleotide reductase displays autoproteolytic activity, cleaving the R1 peptide (Mao et al., 1989, 1992). Cys84 and Cys87 of CMVP conform to the "CysXaaXaaCys" motif common to many oxidoreductases (Holmgren, 1989; Siedler et al., 1993). For CMVP, it is not known if the two pairs of cysteines are in close proximity, or if the protease itself performs an oxidoreductase-type function in the infected cell.

The loss of proteolytic activity due to disulfide bond formation, coupled with the observation that GSSG is capable of inhibiting CMVP, may have some significance to the regulation of CMVP activity during viral infection. In cells, the ratio of GSH/GSSG is normally 100−400 (Gilbert, 1995; Meister, 1995). The levels of GSH and GSSG can change in response to diverse factors, such as nutritional status, hormones, and oxidative stress (Gilbert, 1995). It has been proposed that a change in the glutathione redox status could provide a regulatory signal to specific proteins which are modulated by thiols and disulfides (Gilbert, 1995). Increasing evidence suggests that viral infection can affect the cellular GSH/GSSG ratio. Infection of Vero cells by herpes simplex virus type 1 (HSV-1) is followed by a rapid depletion of endogenous GSH, which decreases ∼3-fold within the first hour, and ∼6-fold by 24 h (Palamara et al., 1995). Conversely, agents that increase intracellular levels of GSH have been shown to exhibit antiviral effects; exogenously supplied GSH inhibits HSV-1 replication in the late stages of the virus life cycle, possibly maturation of virus particles (Palamara et al., 1995). Thus, modulation of the cellular redox status during viral infection may be part of a mechanism used to initiate and terminate different stages of the viral life cycle.

We and others have shown that the 85- and 80-kDa precursors of CMVP accumulate during virus infection, but not during transient transfection, suggesting that their proteolytic conversion into the 30-kDa mature protease is inhibited in the infected cell (Welch et al., 1993; Jones et al., 1994). If HCMV is similar to HSV-1 (both are members of the family Herpesviridae) in causing an overall decrease in intracellular GSH levels, inhibition of CMVP activity by disulfide bond formation may occur in the infected cell. Activation of CMVP could then be achieved, for example, upon transport to a more reducing compartment of the cell (e.g., the site of capsid formation in the nucleus). The present data on the regulation of CMVP activity by flavins and GSSG, together with the role of GSH in viral infection, suggest that change in the redox state of the cell is a plausible mechanism for regulation of CMVP activity during infection.

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