# Evidence of a Conformational Change in the Human Cytomegalovirus Protease upon Binding of Peptidyl-Activated Carbonyl Inhibitors

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ABSTRACT: A series of *N*-*tert*-butylacetyl-L-*tert*-butylglycyl-L-$N^{\gamma}$,$N^{\gamma}$-dimethylasparagyl-L-alanyl-derived inhibitors (trifluoromethyl ketone **1**, pentafluoroethyl ketone, **2**, methyl ketone **3**, and α-ketoamide **4**, with respective $K_I$ values of 1.1, 0.1, 2100, and 0.2 $\mu$M) of the human cytomegalovirus protease were used to study the effect of binding of peptidyl inhibitors on the intrinsic fluorescence and CD properties of the enzyme. In the presence of saturating concentrations of compounds **1**, **2**, and **4**, an identical blue shift in the fluorescence maximum of the enzyme upon specific tryptophan excitation was observed relative to that of the free protease. In the case of the methyl ketone **3**, whose inhibition of the enzyme does not involve formation of a covalent adduct as evidenced by $^{13}C$ NMR studies of carbonyl-labeled inhibitors, the blue shift in the emission was also observed. For both compounds **1** and **2** which exhibit slow-binding kinetics, the observed rate constants for the slow onset of inhibition of substrate hydrolysis correlate well with the $k_{obs}$ values of the time-dependent change in the emission spectra. Studies employing a double mutant of HCMV protease Ala143Gln/Trp42Phe identified Trp-42 as the principal fluorescence reporter. Taken together with information provided by our recent elucidation of the crystallographic structure of the enzyme [Tong, L., Qian, C., Massariol, M.-J., Bonneau, P. R., Cordingley, M. G., & Lagacé, L. (1996) *Nature 383*, 272], these observations are consistent with the inhibition of HCMV protease by peptidyl ketones involving a conformational change of the protease. A mechanism involving a $k_{on}$ limited by dehydration of the hydrated species, followed by rapid ligand binding and a conformational change prior to covalent adduct formation, is proposed for activated inhibitors such as **1** and **2**.

The human cytomegalovirus (HCMV)[1] is a highly prevalent pathogen which poses a serious risk to immunocompromised individuals, notably AIDS patients, organ transplant recipients, and neonates who acquire the infection congenitally (Mocarski, 1995; Britt & Alford, 1995). Typical of members of the herpes virus family, HCMV encodes a unique protease involved in capsid assembly whose activity is essential to the production of infectious virions (Gibson et al., 1995; Gao et al., 1994; Preston et al., 1983; Matusick-Kumar et al., 1995). The enzyme is responsible, late in the viral cycle, for the processing of the assembly protein whose function is analogous to that of the "scaffolding" protein of bacteriophages (Casjens & King, 1975). In the case of HCMV's close homologue HSV-1, failure to process the assembly protein results in the accumulation of aberrant, noninfectious capsids (Preston et al., 1984).

The full-length HCMV protease contains 708 amino acids encoded by the UL80 gene which is coterminal with the UL80.5 (Gibson et al., 1995). The UL80.5 ORF codes for the assembly protein precursor and is in frame with the 373 carboxyl-terminal amino acids of the protease. As a result, the enzyme can process its own C-terminus at a site identical to that of its substrate (maturation or M-site). The protease also undergoes self-processing at a release (R) site near its aminoterminus. This cleavage liberates the 256 amino acid catalytic domain which possesses significant sequence homology with other herpes proteases.

The distinct nature of HCMV protease has recently been underlined by the elucidation of its X-ray crystallographic structure (Tong et al., 1996; Qiu et al., 1996; Shieh et al., 1996; Chen et al., 1996). The enzyme has revealed itself to be a protein with a backbone fold unique among serine proteases and, possibly, among proteins known to date (Tong et al., 1996). These studies have also unequivocally confirmed the status of the enzyme as a serine protease with a unique catalytic triad in which the third member is a histidine as opposed to the normally present aspartate or glutamate residue. Its existence in solution as a dimer which is believed to be the sole active species (Darke et al., 1996; Margosiak et al., 1996) also sets it apart from other serine proteases.

Our medicinal chemistry efforts toward inhibitors of HCMV protease have generated a number of potent activated carbonyl compounds (Ogilvie et al., in preparation), among them the peptidyl trifluoromethyl ketone (TFMK) **1**, its pentafluoroethyl (PFEK) homologue **2**, and the α-ketoamide

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[1] Abbreviations: HCMV, human cytomegalovirus; HSV, herpes simplex virus; TFMK, trifluoromethyl ketone; PFEK, pentafluoroethyl ketone; HLE, human leucocyte elastase; PPE, porcine pancreatic elastase; DTT, dithiothreitol; NMR, nuclear magnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; TCEP, tris(2-carboxyethyl)phosphine; DMSO, dimethyl sulfoxide; HPLC, high-pressure liquid chromatography; CD, circular dichroism.

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**4**. Molecules of this type are classical inhibitors of serine proteases (Imperiali & Abeles, 1986; Mehdi, 1993) which act in a competitive fashion by reversibly forming covalent hemiketal adducts with the active site serine hydroxyl. In the course of NMR studies aimed at observing the association of a peptidyl TFMK with HCMV protease, gross changes in the $^1$H NMR spectrum of the enzyme upon incubation with the inhibitor were observed. This suggested that binding of the TFMK resulted in a significant alteration in the structure of the protease and prompted us to investigate the possibility of a conformational change being associated with the inhibition process.

The proposal of a structural reorganization within a serine protease in the course of inhibition by activated carbonyl compounds is not without precedent and remains a subject of debate, notably in the case of TFMKs. A rate-determining conformational change for the inhibition of human leucocyte elastase (HLE) by peptidyl TFMKs has been put forward to account for kinetic isotope effects of unity for $k_{on}$ (Stein et al., 1987a). It has also been proposed, for catalysis by HLE, that such a change can only be induced by substrates of sufficient length and that this change is also a requirement for optimal hydrolytic activity (Stein et al., 1987b,c). A conformational change following hemiacetal formation has been implicated in the inhibition of chymotrypsin by the aldehyde chymostatin (Stein & Strimpler, 1987). Finally, spectroscopic evidence for the acylation step of catalysis by chymotrypsin being accompanied by an overall change in structure has also been provided (Fink & Wildi, 1974).

In contrast, comparison of the X-ray crystallographic structures of peptidyl TFMK complexes of chymotrypsin to that of the native enzyme failed to reveal any appreciable differences, leading to the conclusion that no conformational change accompanies inhibitor binding (Brady et al., 1990). Similar studies involving porcine pancreatic elastase and its covalent complexes with a peptidyl TFMK (Takahashi et al., 1988) as well as other activated carbonyl inhibitors have likewise led to the conclusion that this protease is a "lock and key" enzyme (Edwards et al., 1992).

The essential role of HCMV protease in viral replication makes it an attractive target for the development of new therapeutic agents. The novel nature of this enzyme, which clearly sets it apart from other serine proteases, also constitutes a strong incentive for further investigation of its structure, catalysis, and inhibition. In the present work, we provide evidence that the inhibition of HCMV protease by peptidyl carbonyl compounds is accompanied by a structural change in the enzyme. It is hoped that the observations described herein will contribute to gaining a clearer picture of the structural aspects of this inhibition process and will assist in both the development of potent inhibitors and in furthering our understanding of this novel enzyme.

## MATERIALS AND METHODS

*Preparation of Enzymes.* With the exception of those involving the HCMV protease double mutant Ala143Gln/Trp42Phe, all studies were carried out using the mutant Ala143Gln, which eliminated the problem of autoproteolysis (Pinko et al., 1995). The coding sequence for the catalytic domain of HCMV protease was amplified from DNA obtained from HCMV (strain AD169) infected cells employing the polymerase chain reaction technique using oligonucleotide primers specific for the N- and C-terminal sequences of the enzyme. The amplified DNA was then cleaved with *Nde*I and *Bam*HI and inserted into the *Nde*I/*Bam*HI restriction sites of the expression plasmid pET17b. The recombinant plasmid was used as a template to introduce substitutions at Trp-42 and Ala-143 by site-directed mutagenesis through the method of overlap extension (Higuchi et al., 1988; Ho et al., 1989). The resulting constructs coding for the single and double mutants of the protease were verified by DNA sequencing.

The plasmids were transformed into *Escherichia coli* strain BL21(DE3) pLysS for protein expression. The *E. coli* clones containing mutant proteases were grown at 37 °C in cycle growth medium containing ampicillin (100 $\mu$g/mL) and chloramphenicol (34 $\mu$g/mL). When the $A_{600}$ reached 0.5, the cultures were cooled to 20 °C and IPTG was added (0.1 mM). The induction was performed at 20 °C for 5 h after which time the cultures were centrifuged at 5500*g* for 10 min and the cell pellet was resuspended in lysis buffer (35 mL/L of culture; 50 mM Tris-HCl, pH 7.8, 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) and stored at −80 °C.

The recombinant proteases were purified by the same procedure. The bacterial pellet was thawed at 37 °C to lyse the cells, and all subsequent operations were performed at 4 °C. The cell lysate was sonicated to reduce its viscosity and cleared by centrifugation at 13 000 rpm for 30 min in a JA17 rotor. Streptomycin sulfate (5% solution, w/v) was added to the lysate to a concentration of 1% and the soluble material recovered by centrifugation as describe above, followed by the addition of ammonium sulfate to the supernatant to 45% saturation. Precipitated proteins were recovered by centrifugation, and the protein pellet was suspended in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1 mM EDTA), dialyzed against this buffer, and then applied onto a 6 mL Resource Q (Pharmacia) anion-exchange column. Proteins were eluted with a 0−0.5 M NaCl linear gradient in buffer A. Fractions containing the protease were pooled, dialyzed against buffer B (20 mM acetate, pH 5.0, 1 mM DTT, 0.1 mM EDTA), and loaded onto a 6 mL Resource S (Pharmacia) cation-exchange column. The protein was eluted with a 0−0.5 M NaCl linear gradient in buffer B. The procedure yielded 50 mg of purified protease/L of *E. coli* culture. The purities of HCMV protease mutants Ala143Gln and Ala143Gln/Trp42Phe were estimated to be >99% by densitometry of Coomassie stained SDS−PAGE gels.

*Synthesis of Inhibitors.* Detailed procedures for the synthesis and purification of peptidyl methyl ketone **3** and its corresponding alcohol **5**, as well as characterization of intermediates and the final products (including [$^{13}$C]-**3**), are provided in the Supporting Information (see paragraph at end of paper regarding Supporting Information). Methyl ketone **3** was prepared as a single diastereomer whereas alcohol **5** consists of an unassigned 65:35 mixture of epimers at the alcohol center. The activated carbonyl inhibitors **1**, **2**, [$^{13}$C]-**2**, **4**, and **6**, which are highly prone to epimerization at the alanyl α-carbon, are diastereomeric mixtures of unknown composition and are likely fully epimerized. Details of the preparation of these inhibitors will be published shortly (Ogilvie et al., in preparation). Their physical properties are provided in the Supporting Information. The purity of all inhibitors was established by HPLC analysis and by high-field NMR.

*Enzyme Assays.* Hydrolysis of the internally quenched peptide substrate anthranilamide-VVNA-SSRLY(3-$NO_2$)R-

OH was monitored at 30 °C by the increase in fluorescence intensity upon cleavage of the Ala−Ser bond (for anthranilamide $\lambda_{ex} = 312$ nm and $\lambda_{em} = 415$ nm, slit widths varied from 2.5 to 5 nm). The incubation mixture was composed of 50 mM Tris-HCl, pH 8.0, 0.5 M $Na_2SO_4$, 50 mM NaCl, 0.1 mM EDTA, 1 mM TCEP, 3% DMSO (v/v), and 1 $\mu$M HCMV protease and contained 4 $\mu$M substrate (well below the estimated $K_M$ value of 760 $\mu$M with $k_{cat}/K_M = 260$ $M^{-1}$ $s^{-1}$). Large excesses of inhibitor at concentrations typically 50−100-fold their $IC_{50}$ values (100 $\mu$M for **1** and **6**, 50 $\mu$M for **2** and **4**) were chosen to completely saturate the enzyme at equilibrium before significant substrate depletion occurred. In the case of compounds **3** and **5**, solubility limited the concentrations used to 5 mM. The reaction was initiated by the addition of the enzyme and was monitored until complete inhibition was achieved. The apparent rate constants of the processes were determined by submitting the curves to first-order nonlinear regression analysis using the kinetic software GRAFIT (Erithacus Software Ltd., 1989−1992, Version 3.0, R. J. Leatherbarrow, London, U.K.).

*NMR Experiments.* Proton-decoupled $^{13}C$ NMR spectra were recorded at either 100.61 or 150.90 MHz on Bruker AMX 400 and DMX 600 spectrometers, respectively. In the former case, spectral conditions were 236 ppm spectral width, 10 $\mu$sc pulse width, 2 s repetition rate, and 15 000 transients acquired. At the higher field, the conditions were 120 ppm spectral width, 5 $\mu$s pulse width, 2.5 s repetition rate, and 33 600 transients acquired. In both cases, 32K data points were acquired, a 3 Hz line-broadening factor was applied, experiments were carried out at 300 K, and internal DMSO, assigned a chemical shift of 39.50 ppm, was used as the reference.

Samples of inhibitor alone were prepared by adding solutions of labeled ketones in DMSO-$d_6$ (30 $\mu$L) to buffer (500 $\mu$L) consisting of 50 mM deuterated Tris, 1.0 mM deuterated EDTA, 5.0 mM deuterated DTT, 50 mM NaCl, and 0.5 M $Na_2SO_4$ containing $D_2O$ (70 $\mu$L) for lock purposes. Final inhibitor concentrations of [$^{13}C$]-**2** and [$^{13}C$]-**3** were 1.3 and 3.3 mM, respectively, and a pH of 6.9 was verified. Samples containing enzyme were prepared in a similar fashion using buffer containing 0.76 mM HCMV protease. The final concentration of protease was 0.72 mM, and the samples were incubated for at least 1 h prior to the acquisition of spectra. Molar excesses of 2.2- and 5-fold of inhibitor over the protein were used for inhibitors [$^{13}C$]-**2** and [$^{13}C$]-**3**, respectively.

*Fluorescence Studies.* Fluorescence measurements were performed in 1 mL quartz cuvettes using a Perkin-Elmer LS-50B spectrofluorometer and are uncorrected since only relative comparisons were analyzed. The emission spectra of HCMV proteases were recorded upon excitation at 280 or 295 nm (emission slit width varied from 5 to 7 nm). Excitation at 280 nm was preferred since overlap of the residual Raman scatter band and the emission band was minimal. The incubation buffer was identical to that employed in the enzyme assay, and the final concentrations of both enzyme and inhibitor were equal to those of assay conditions (see above). Scans were obtained at 30 °C after 1.5 h preincubation of the samples at that temperature. In all cases, blank samples containing the inhibitor alone in buffer were scanned to verify the absence of any undesirable interference, and the spectra were subtracted from those of samples containing enzyme.

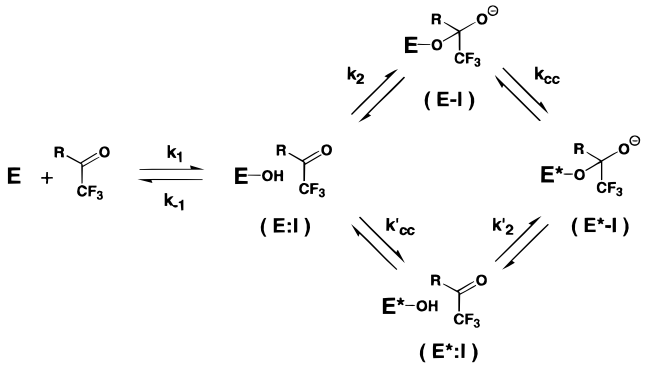



FIGURE 1: Possible mechanisms of inhibition of HCMV protease by peptidyl TFMK inhibitors which involve a conformational change (E → E*) of the enzyme.

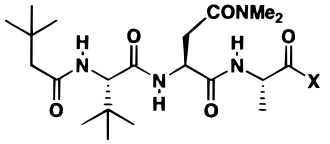
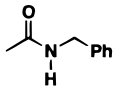
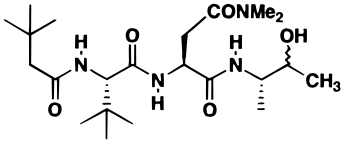
Identical buffer conditions were employed for monitoring the rates of the fluorescence shifts in the presence of slow-binding inhibitors with the emission monochromator set at 320 nm (*i.e.*, about 20 nm lower than the emission maximum of the free enzyme). Care was taken to ensure that the final enzyme and inhibitor concentrations were identical to those used in monitoring the onset of inhibition under assay conditions (see above). The reaction was initiated by the addition of enzyme to a preincubated sample of the inhibitor at 30 °C. The time-dependent changes in emission intensity at 320 and 360 nm were monitored until the fluorescence reached its new equilibrium, and the apparent rate constants, $k_{obs}$, were obtained as described above.

*Circular Dichroism Studies.* CD spectra were recorded in a JASCO J-720 spectropolarimeter at ambient temperature under the same buffer conditions as described for the enzyme assay (see above). The cell path length for the near-UV spectra was 10 mm. An enzyme concentration of 13 $\mu$M (based on an $\epsilon_{280nm}$ of 28 420 $M^{-1}$ for HCMV protease) and inhibitor concentrations of 130 $\mu$M were used. The spectra presented are an average of ten scans after subtraction of a buffer spectrum which was also an average of ten scans. Mean-residue ellipticity values ($\theta$) are expressed for all wavelengths as deg·$cm^2$/dmol.

## RESULTS AND DISCUSSION

On the basis of our initial observations of a possible alteration of enzyme conformation by proton NMR, it was our hypothesis that the inhibition of HCMV protease by peptidyl-activated carbonyl compounds occurs through either of two mechanisms similar to those previously proposed for the inhibition of HLE by peptidyl TFMKs (Stein et al., 1987a). As outlined in Figure 1, the enzyme and inhibitor, the latter in its ketonic form after a dehydration step, initially form a relatively weak Michaelis-like complex (E·I) mediated by interactions between the peptidic chain and the binding pocket of the protease. This is then followed either by a conformational change ($k'_{cc}$) requisite for attack of the electrophilic carbonyl by the active site serine ($k'_2$) or by hemiketal formation ($k_2$) which then induces a structural reorganization of the protease ($k_{cc}$). In either case, a consequence of such an overall mechanism would be that the free enzyme or initial enzyme−inhibitor complex (E·I) and the complex after hemiketal formation and the putative conformational change (E*−I) would differ in structure and, as a consequence, should exhibit distinct biophysical behavior as detected by fluorescence and circular dichroism. We were also interested in addressing the question of whether or not

Table 1: Inhibition of HCMV Protease by Peptidyl Ketones[a]

| Inhibitor[b] | | $IC_{50}$ (μM) |
|---|---|---|
|  | **1**: X = $CF_3$ | 1.1 |
| | **2**: X = $CF_2CF_3$ | 0.1 |
| | **3**: X = $CH_3$ | 2100 |
| | **4**: X =  | 0.2 |
|  | **5** | 7500 |
|  | **6** | 1.8 |

[a] Protease activity was determined by a fluorogenic assay as described in Materials and Methods with the following modifications: [HCMV protease] = 100 nm, [substrate] = 5 μM, [**1**, **2**, **4**, and **6**] = 300 μM−60 nM, [**3** and **5**] = 5 mM−15 μM, 0.05% (w/v) casein, substrate added after 5 h preincubation of inhibitor and enzyme at 30 °C. [b] Inhibitors **1**, **2**, **4**, and **6** are at least partially epimerized at the alanyl α-center. MK **3** is a single diastereomer. Alcohol **5** consists of unassigned 65:35 mixture of diastereomers.

the putative conformational change be rate-limiting to the inhibition process and, if so, be implicated in the slow-binding kinetics associated with activated carbonyl inhibitors of the present type.

*Design of Inhibitors and NMR Investigations.* To assess our model, two potent inhibitors of HCMV protease, the peptidyl TFMK **1** and its pentafluoroethyl analogue **2**, were chosen. The medicinal chemistry efforts which led to the peptidic structure will be published shortly (Ogilvie et al., in preparation). The potency of such activated carbonyl compounds toward their serine protease targets is due, in large part, to the highly electrophilic nature of their carbonyl centers. The strongly electron-withdrawing perfluoroalkyl group of the ketone allows for facile attack by the active site serine and the formation of a stable hemiketal adduct. The existence of such tetrahedral adducts has been evidenced by NMR investigations involving TFMK adducts of chymotrypsin (Liang & Abeles, 1987) and has been confirmed by crystallographic studies of TFMK inhibitor complexes of chymotrypsin (Brady et al., 1990) and porcine pancreatic elastase (PPE) (Takahashi et al., 1988). Peptidyl α-ketoamides similar to inhibitor **4** have also been shown by X-ray crystallography to form tetrahedral adducts in the case of elastase (Edwards et al., 1992). Ample evidence also exists that it is the nonhydrated keto forms of such compounds which are the active species. The inhibition of HCMV protease by inhibitors **1**, **2**, and **4** presumably occurs in a similar fashion.

Central to the present study was to identify an inhibitor which would complex to the enzyme in a manner analogous to **1** and **2** *without* forming the covalent hemiketal adduct. The peptidyl methyl ketone (MK) **3** appeared to best satisfy these requirements. The inhibition constants for all compounds studied herein are given in Table 1. As one might expect, **1** and **2** exhibit comparable potencies whereas the MK **3** is 3 orders of magnitude less active. Such large differences in inhibitory activity between peptidyl TFMKs and their corresponding MKs have been reported for chymotrypsin (Imperiali & Abeles, 1986), HLE (Edwards et al., 1992), and acetylcholinesterase (Allen & Abeles, 1989) and are cited as evidence of the importance of carbonyl activation in permitting a stable covalent adduct to form (or, otherwise stated, the inability of a methyl ketone to do so). The great differences in reactivity and hemiacetal stability between TFMKs and MKs are reflected in their respective degrees of hydration in aqueous solution. In the case of the former, the hydrate/ketone ratio for peptidyl compounds has been estimated to be as high as 4500 (Brady & Abeles, 1990) whereas nonfluorinated analogues are essentially nonhydrated. The comparable potencies of alcohol **5** and MK **3** are also consistent with the absence of covalent adduct formation during inhibition.

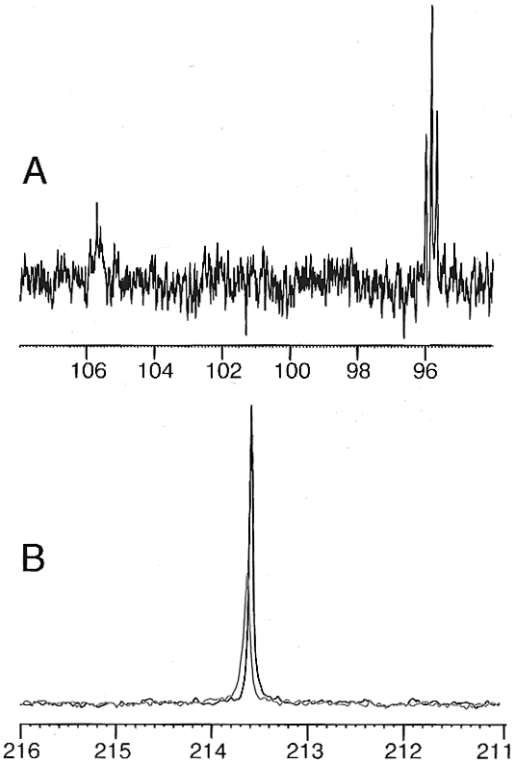



FIGURE 2: Sections of the $^{13}C$ NMR spectra of (A) 1.6 mM PFEK [$^{13}C$]-**2** in the presence of 0.72 mM HCMV protease. The signals at 105.7 and 95.8 ppm correspond to the bound (ionized hemiketal) and free inhibitor, respectively. (B) 3.6 mM methyl ketone [$^{13}C$]-**3**. The trace in black corresponds to the free inhibitor whereas that in red corresponds to the inhibitor in the presence of 0.72 mM HCMV protease. See Materials and Methods for spectral conditions and sample compositions.

In order to obtain more direct evidence that inhibition of the protease by the methyl ketone **3** does not involve covalent adduct formation, [$^{13}C$]carbonyl-labeled samples of **3** and PFEK **2** were prepared for NMR studies (Figure 2). The $^{13}C$ spectrum of the labeled PFEK [$^{13}C$]-**2** alone presented a sharp signal at 95.8 ppm corresponding to the hydrated form of the inhibitor and which exhibited the expected triplet multiplicity due to coupling to the adjacent fluorine atoms ($^2J_{C-F}$ = 24 Hz). Incubation of a 2.2-fold excess of the inhibitor and HCMV protease resulted in the appearance of a new signal at 105.7 ppm (Figure 2A). The broadened nature of the resonance, which masked any multiplicity, was consistent with a protein-associated species which we assign to be the ionized hemiketal adduct formed between [$^{13}C$]-**2** and the enzyme which is in slow exchange with the free inhibitor. These results are nearly identical to those described for similar $^{13}C$ NMR studies involving the complex of a

labeled TFMK inhibitor and α-chymotrypsin (Liang & Abeles, 1987) on which we base our signal assignments. Similar observations were also noted for investigations probing the interaction of [$^{13}$C]carbonyl-labeled arachidonic acid and phospholipase $A_2$ (Trimble et al., 1993).

The analogous experiments were also carried out for the labeled MK [$^{13}$C]-**3**. In the absence of protease, the $^{13}$C spectrum of the inhibitor presented a sharp singlet at 213.6 ppm. As anticipated, no hydrated form was detected. When a 5-fold excess of labeled ketone [$^{13}$C]-**3** was incubated with the protease, the signal was clearly altered (Figure 2B). The new resonance was broadened and of decreased intensity (as verified through intensity calibration using the DMSO peak). A very small but dicernible downfield shift (>0.05 ppm) was also noted.

No signal in the 100 ppm range, where one would expect a hemiketal signal to occur, could be detected, which eliminates the possibility of slow exchange between free [$^{13}$C]-**3** and a covalent adduct. The observed line broadening, and the absence of any other broadened signal upfield of the observed peak, is indicative of rapid exchange between the free inhibitor and a protease-bound species with a chemical shift very close to that of the unbound ligand, likely downfield from it if the slight shifting of the new peak is considered. Since only a carbonyl center could give rise to a $^{13}$C resonance in this parts per million range, we conclude that MK **3** is complexed by HCMV protease as a ketone which is not attacked by the active site serine.

Clear evidence that MK **3** does indeed inhibit the enzyme by binding to the active site was provided by proton NMR competition studies in which differential line broadening was observed for the inhibitor signals in the presence of the protease (data not shown; a detailed account of our proton NMR studies involving HCMV protease will be published shortly). When PFEK **2** was subsequently added to the sample, the signals of MK **3** were restored to their uncomplexed peak heights due to the displacement of the methyl ketone from the enzyme by the much more potent activated carbonyl analogue.

*Changes in Fluorescence Emission.* Fluorescence spectroscopy is a well-established method of probing protein structure and has often been employed to monitor alterations in the structure of a serine protease with respect to solvent composition, denaturating agents, temperature, and pH. The activating effect of kosmotropic agents on HSV-1 protease has been probed through the emission spectrum of the enzyme (Hall & Darke, 1995) and through the use of a fluorescent probe (Yamanaka et al., 1995). We are, however, unaware of any studies which utilize the intrinsic fluorescent properties of a serine protease to probe the question of a conformational change occurring upon peptidyl inhibitor binding.

HCMV protease contains 16 excitable chromophores including three tryptophan residues. Upon excitation at 280 nm, a fluorescence emission with a maximum at 348 nm was observed (Figure 3). Excitation at 295 nm resulted in an essentially identical spectrum, indicating that the fluorescence arises from specific excitation of the tryptophans. Upon incubation of the protease with saturating concentrations of the inhibitors **1** (TFMK), **2** (PFEK), or **4** (α-ketoamide), marked blue shifts in the emission maxima to 339 nm were observed, accompanied by small increases in intensity (Figure 4). This suggests that a key fluorophore(s) find(s) itself in a more hydrophobic environment upon

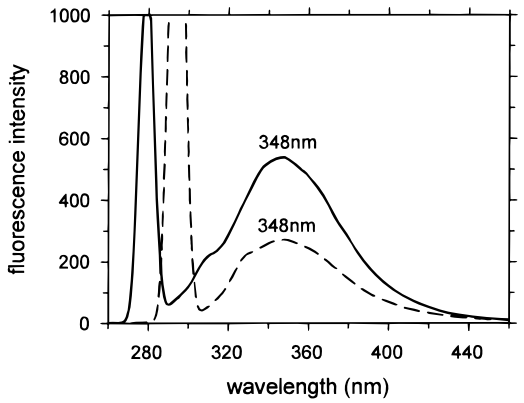



FIGURE 3: Fluorescence spectra of HCMV protease upon excitation at 280 nm (—) and 295 nm (---) revealing the specific excitation of tryptophan residues.

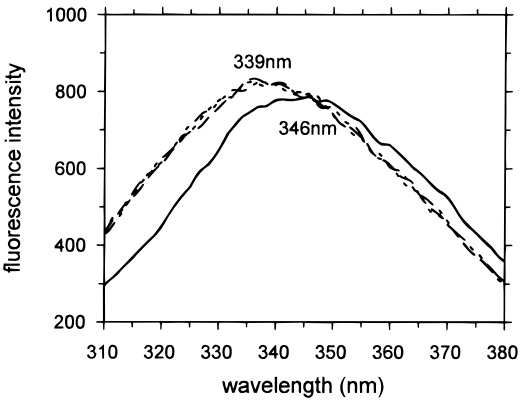



FIGURE 4: Changes in the fluorescence spectrum of HCMV protease in the presence of peptidyl-activated carbonyl inhibitors. All the spectra shown henceforth were acquired after excitation at 280 nm using samples containing 1 $\mu$M enzyme under the conditions described in Materials and Methods. Curves: HCMV protease alone (—) and in the presence of saturating concentrations of TFMK **1** (---), PFEK **2** (— —) and α-ketoamide **4** (-·-).

inhibitor binding. The great similarity between the fluorescence bands of the three E—I complexes strongly suggests that the same tryptophan perturbation is operative for all. The effect of TFMK **6** on the fluorescence properties of the protease was also investigated and provided identical results (data not shown). The fact that there are the same changes in the emission spectrum of the enzyme upon binding of this particular inhibitor is important; it demonstrates that the same alteration of the tryptophan environment may be induced by an inhibitor which closely mimics the natural substrate (M-site) of the enzyme and is not a phenomenon particular to the optimized peptidic chain of compounds **1**—**5**.

Central to the present study was the experiment involving MK **3**. Solubility limitations obliged us to use an inhibitor concentration of 5 mM at which approximately 70% of the enzyme was complexed. In this case, a shift in the emission maximum from 348 to 340 nm was observed although a pronounced decrease in the intensity of the band was also recorded (Figure 5). We ascribe this decrease in fluorescence to absorption by the ketone moiety of **3**. Despite the fact that it is very weak, the forbidden n $\rightarrow \pi^*$ transition of the carbonyl functionality [$\lambda_{max} = 282$ nm (methanol), $\epsilon = 46$] becomes a significant factor at the high inhibitor concentration employed and contributes sizably to the absorption of the excitation energy by the sample. In spite of this, after adjustment of the scales of the spectra, the obvious presence of the blue shift in the emission maximum, as well as the

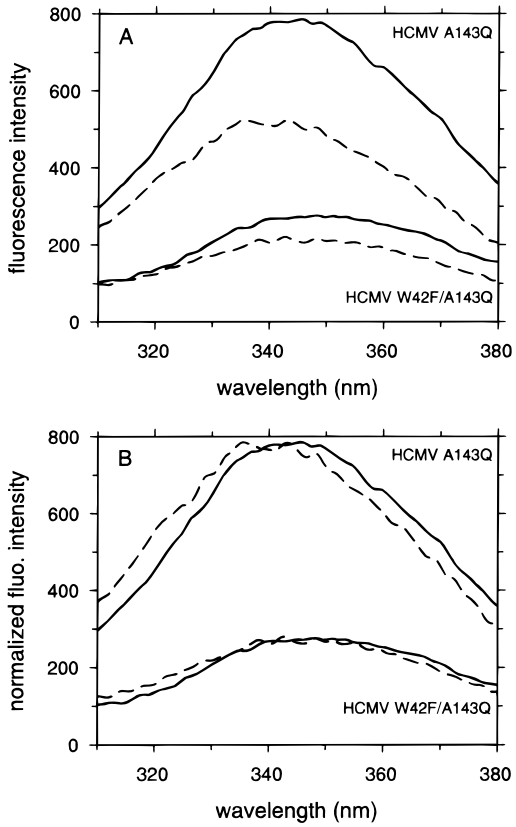



FIGURE 5: Fluorescence spectra of (A) HCMV proteases Ala143Gln and double mutant Ala143Gln/Trp42Phe alone (—) and in the presence of 5 mM MK **3** (---). (B) Same spectra shown after the adjustment of scales.

similarity of the spectrum to those of the protease-containing activated carbonyl inhibitors, becomes clearly evident. A control experiment was also carried out employing a MK **3** concentration of 50 $\mu$M where, as one would anticipate, essentially no change in the emission spectrum was observed (data not shown). It was at comparable concentrations that the much more potent inhibitors **1**, **2**, **4**, and **6** elicited the full spectral changes. To further verify the influence of the ketone moiety on the fluorescence intensity of the protease, a second control experiment was performed in which acetone, to a millimolar concentration, was added to a sample of the enzyme alone. As expected, a similar decrease in the emission intensity was observed; however, no shift in the maximum was apparent after adjustment of the scales.

The X-ray crystal structure of HCMV protease reveals that, of the three tryptophans present in the enzyme, only the residue at position 42 is in any proximity to the active site, the other two being located on the opposite surface of the protein. Reasoning that it was this fluorophore whose immediate environment was most likely to be perturbed upon binding of the inhibitors, a double mutant of HCMV protease Ala143Gln/Trp42Phe in which this tryptophan is replaced by a phenylalanine residue was studied. This enzyme was found to exhibit catalytic activity comparable to that of the single mutant and, in addition, to be inhibited by peptidic-activated carbonyl compounds in a similar fashion.

Upon excitation of the double mutant alone at 280 nm under the standard conditions, an emission band centered at 348 nm was observed which, as one would anticipate, was less intense than that observed for the single mutant. In the presence of a saturating concentration of the TFMK **1**, no shift in the emission maximum was observed although the

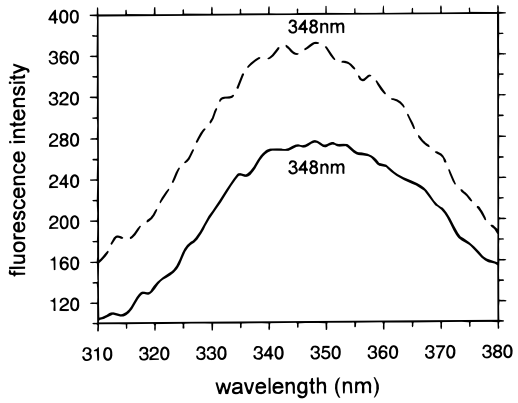



FIGURE 6: Fluorescence spectra of HCMV protease double mutant Ala143Gln/Trp42Phe alone (—) and in the presence of a saturating (100 $\mu$M) concentration of TFMK **1** (---).

band was more intense (Figure 6). Identical results were obtained using PFEK **2** and α-ketoamide **4** (data not shown). Similarly, no significant shift in the fluorescence spectrum was observed when the maximal concentration of MK **3** was added to the enzyme. In this case, the emission band was weaker and the coincidence of the traces was, again, more apparent after the spectra were rescaled (Figure 5). These data indicate that tryptophan-42 is indeed the principal fluorescent reporter.

An important fact allows us to conclude that the observed changes in fluorescence were not simply due to the direct interaction of inhibitor and fluorophore within a rigid binding site on the enzyme. The report of a low-resolution X-ray structure of an HCMV protease-iodinated tetrapeptidyl aldehyde complex (Chen et al., 1996) is in accord with our own structural investigations (manuscript in preparation) which clearly indicate that the aromatic side chain of Trp-42 is not in close proximity to the region occupied by such inhibitors and is separated from it by a mobile loop (Figure 10 and discussion below). In view of this and our present results, we believe that the inhibition of HCMV protease by peptidyl carbonyl compounds involves a structural reorganization of an enzyme−inhibitor complex. In addition, the ability of the inhibitor to form a covalent adduct with the active site serine is not requisite to inducing the conformational change. This transition is most likely elicited by the establishment of contacts between the peptidic portion of the inhibitor and the protease binding sites.

*Changes in Circular Dichroism.* Circular dichroism spectropolarimetry is another commonly used tool for examining changes in the secondary and tertiary structure of proteins. In the latter case, structural changes may be detected in the near-UV region where absorption due to aromatic amino acids occurs. When the CD spectrum of HCMV protease alone and in the presence of TFMK **1** and PFEK **2** was compared, a significant difference was observed (Figure 7). Moreover, the two latter traces were coincident, indicating that similar changes in the tertiary structure of the protease occurred upon binding of the inhibitors. The experiment involving MK **3** was precluded owing to the compound's own optical activity at the high concentration required. The CD spectra of the HCMV protease single mutant (Ala143Gln) and the double mutant Ala143Gln/Trp42Phe, both in the absence of any inhibitor, were found to be similar, suggesting that Trp-42 is conformationally mobile in the free enzyme, as supported by the X-ray data (Tong et al., 1996). Studies involving TFMK **1** and the

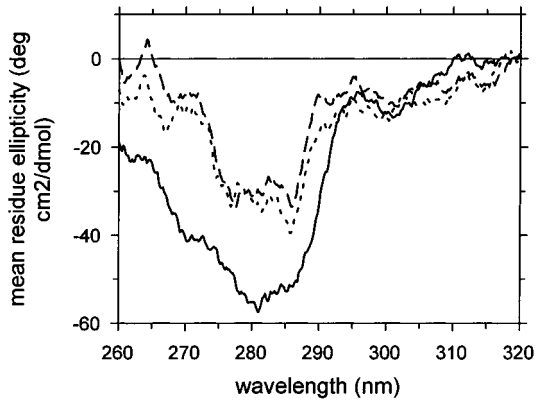



FIGURE 7: Near-UV CD spectra of HCMV protease alone (—) and in the presence of saturating TFMK **1** (— —) and PFEK **2** (---).

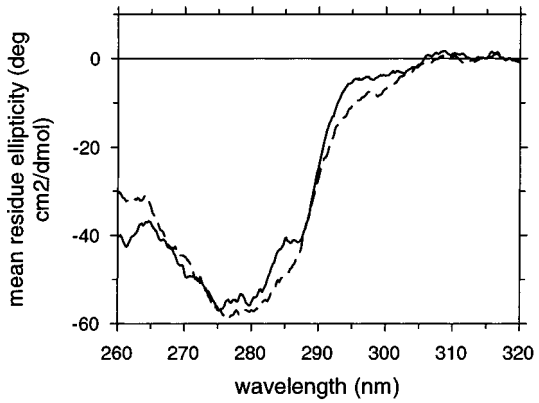



FIGURE 8: Near-UV CD spectra of HCMV protease double mutant Ala143Gln/Trp42Phe alone (—) and in the presence of TFMK **1** (— —).

HCMV protease double mutant were also carried out and showed no major changes in the CD spectrum upon the addition of the inhibitor (Figure 8). Spectral investigations of the far-UV region were also performed and were hampered by interference due to buffer components at wavelengths below 210 nm. Little change was seen in the spectrum of the single mutant enzyme between 210 and 250 nm upon the addition of inhibitors **1** and **2** (data not shown), suggesting that the conformational transition does not involve substantial motion of the protein backbone.

The results of the CD investigations are fully consistent with both the conformational change occurring upon binding of the inhibitors and the structural transition occurring, at least in part, in the vicinity of tryptophan-42. With this in mind, one may make tentative assignments to the bands seen in the difference spectra shown in Figure 7. The low-magnitude positive ellipticity bands observed at 287 and 295 nm are most likely due to the optical rotation arising from the $^1L_b$ resonances of the indole ring of tryptophan-42. The spectra of the inhibited complexes indicate that the residue becomes immobilized after the conformational change and may come into contact with another region of the protein. The observed blue shifts in the fluorescence spectra corroborate a burying of the indole into a hydrophobic area of the enzyme.

*Kinetics.* Any discussion of a conformational change linked to serine protease inhibition by TFMKs or similar molecules cannot exclude some consideration of the kinetics of the process. It is well documented that the action of such compounds against these enzymes is very often characterized by a slow onset of inhibition. This "slow-binding" phe-

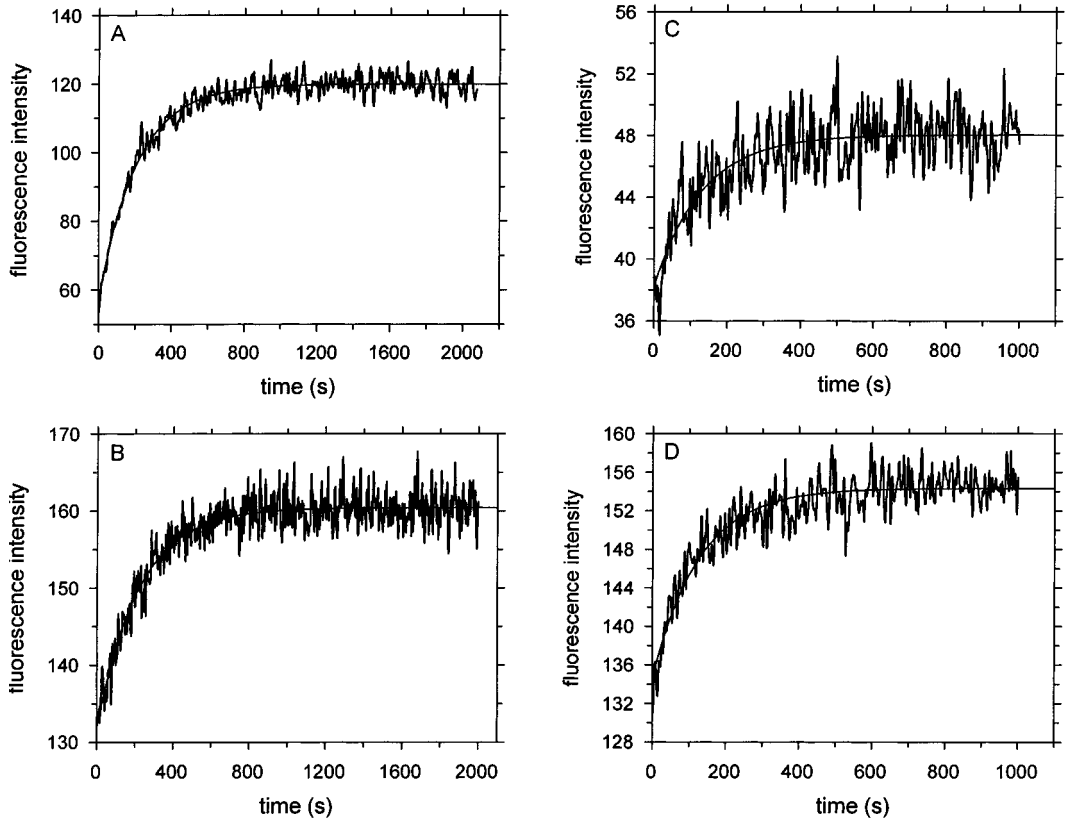



FIGURE 9: Progress curves for the inhibition of HCMV protease by (A) 100 $\mu$M TFMK **1** and (C) 25 $\mu$M PFEK **2**. Fluorescence intensity was monitored at 415 nm (excitation at 312 nm) for solutions containing 1 $\mu$M enzyme and 4 $\mu$M fluorogenic substrate under buffer conditions described in Materials and Methods. Time dependence of the change in fluorescence of HCMV protease in the presence of (B) 100 $\mu$M TFMK **1** and (D) 25 $\mu$M PFEK **2**. Conditions used were identical to those of (A) and (B) except that substrate was not present. Emission intensity was recorded at 320 nm using an excitation wavelength of 280 nm.

nomenon is normally accounted for by the aforementioned fact that, in aqueous solution, TFMKs exist almost exclusively in their hydrated forms (Edwards & Bernstein, 1994, and references cited therein). Since it is the ketone which is the active species, an extremely low effective inhibitor concentration could result in the observed kinetics. On the other hand, a slow conformational change within an enzyme−inhibitor complex in which contacts with the peptidic portion of the inhibitors play a key role has also been put forward to explain slow binding (Stein et al., 1987a). A fundamental question underlying this debate is whether serine proteases are "lock and key" enzymes or whether an "induced-fit" model best applies.

Figure 9A shows the effect of a large excess of TFMK **1** on the hydrolysis of a fluorogenic substrate by HCMV protease, which exhibits the typical slow onset of full inhibition. The effect of compound **1** on the fluorescence emission of the enzyme under identical conditions, but in the absence of substrate, was also followed in time by monitoring the increase in emission intensity at 320 nm (Figure 9B). The identity of the curves is striking and provides telling evidence that the change in protease conformation is directly linked to the inhibition process. Apparent first-order rate constants of $4.8 \times 10^{-3}$ and $4.5 \times 10^{-3}$ $s^{-1}$, respectively, were calculated for the rates of onset of inhibition and change in emission. The analogous experiment was performed using the PFEK **2** (Figure 9c,d), which also showed very good correlation between the two effects. In this case, the onset of inhibition was more rapid with $k_{obs}$ values of $7.7 \times 10^{-3}$ $s^{-1}$ measured for both processes. The changes in emission spectra could also be monitored by the decrease in intensity at 360 nm, which gave comparable values of $k_{obs}$. The inhibitor-induced changes in near-UV CD spectra for compounds **1** and **2** also exhibited similar time dependencies (data not shown). Finally, the effect of the MK **3** was studied using a concentration as high as solubility would permit. As expected for a rapid and reversible, competitive inhibitor, the onset of its effect on substrate hydrolysis was too rapid to be measured, as was the rate of change in protein fluorescence.

## CONCLUSIONS

In the present study, we provide spectroscopic evidence that HCMV protease undergoes a conformational change upon inhibition by peptidyl ketones and that this structural alteration is not associated with the ability of the inhibitor to form a covalent adduct with the active site serine. This latter point is based on studies involving the labeled inhibitor [$^{13}$C]-**3**, which is not attacked by the active site serine after complexation by the enzyme as evidenced by $^{13}$C NMR experiments. In the cases of TFMK **1** and PFEK **2**, the kinetics of the structural change as monitored by fluorescence emission (as well as CD) are of particular interest in that they correlate very well with the slow-binding associated with this class of inhibitors. In view of this correlation, one might be tempted to conclude that the slow binding phenomenon is a consequence of a rate-determining conformational change. It must be considered, however, that the observed kinetics may just as well be a reflection of a slow step preceding a rapid conformational transition which occurs during or after binding of the ketonic inhibitor.

Considering the very close structural similarity shared by the present series of inhibitors **1**−**4**, we find it highly unlikely

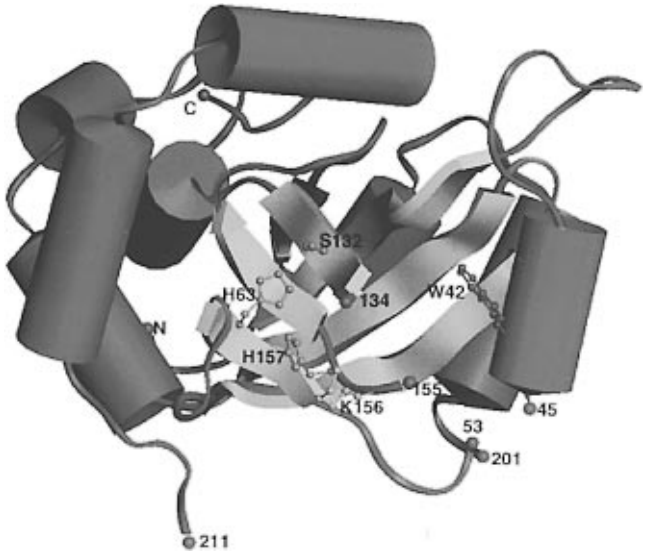



FIGURE 10: X-ray crystallographic structure of HCMV protease (Tong et al., 1996) highlighting the principal fluorescence reporter Trp-42 (blue), junctions of the loops 45−53 and 134−155 (green spheres), and the active site catalytic triad (pink). Bound tetrapeptidyl inhibitors are expected to occupy a region spanning Ser-132 to Lys-156. Indeed, a low-resolution X-ray structure of a bound tetrapeptidyl inhibitor iodinated at P4 (Chen et al., 1996) presents the halogen atom in close proximity to Lys-156 (orange). Tryptophans-179 and -199 are situated on the opposite side of the protein.

that the differing observed rates for the fluorescence changes are a reflection of differing rates of the conformational change for each of the respective compounds. Furthermore, the rates of the transition for all the inhibitors are, most likely, very rapid as in the case of the methyl ketone **3** and the ketoamide **4**. The observation of the structural change induced by MK **3** is of central importance in that it demonstrates that formation of a covalent E−I adduct is not a prerequisite for the conformational transition to occur and effectively rules out "conformational tightening" of a tetrahedral adduct.

As far as the origin of the slow-binding kinetics observed for the activated ketones **1** and **2**, our presumption that the conformational change elicited by them is a very rapid process conflicts with the proposition that a rate-determining structural change is responsible for the slow onset of inhibition (as put forward for elastase; Stein et al., 1987a). It is acknowledged, in the cases of chymotrypsin and elastase, that attack of the active site serine on the activated carbonyl of a bound inhibitor is an extremely rapid step (Brady & Abeles, 1990), as is the association of free ketone and enzyme. Assuming that these facts hold equally true for the present enzyme, our results strongly point to an extremely low effective inhibitor concentration due to ketone hydration being the factor which limits the $k_{on}$ of the inhibition process and being the origin of the slow-binding phenomenon. The fact that α-ketoamide **4** and MK **3** which are nonhydrated in solution both exhibit fast binding is also consistent with this conclusion.

We therefore propose that the lower pathway outlined in Figure 1 best represents the inhibition of HCMV protease by peptidyl-activated carbonyl compounds. After rate-limiting inhibitor binding for which the free ketone concentration is the limiting term (in the cases of hydrated TFMKs and PFEKs), the complexed ligand induces a rapid conformational change mediated by contacts between the peptidic

portion of the inhibitor and binding sites on the protease (E·I to E*·I). This change may be required to properly position the active site residues for facile attack of the carbonyl leading to the formation of a stabilized tetrahedral adduct. A similar proposal that substrate binding is required to engage the catalytic triad of HLE has been put forward (Stein et al., 1987b,c) but differs from our proposed mechanism in that we do not believe the conformational change to be the rate-limiting step.

We again underline the fact that the TFMK **6**, which is a pentapeptide derivative of the natural M-site product of the enzyme, also elicits the same conformational change as observed by emission spectral changes. This leads us to believe that binding of natural substrates by HCMV protease also results in the structural change and that the enzyme's functioning be best described in terms of an induced-fit rather than a lock and key model. It must, however, be emphasized that the distinct nature of the present enzyme be taken into consideration in extrapolating any conclusions to the workings of serine proteases in general. It is of obvious interest that the methods outlined in this work be applied to members of classical serine protease families such as chymotrypsin and elastase, and these investigations are presently being carried out.

While the present observations are largely phenomenological, the identification of the fluorescent reporter residue and the recent elucidation of the crystallographic structure of HCMV protease allow us to make some speculation as to the nature of the conformational change (Figure 10). Tryptophan-42 is situated at the end of helix αA (Tong et al., 1996) adjacent to a missing surface loop (45−53) and lies near a second loop (134−155), both whose electron densities cannot be clearly interpreted in any of the published X-ray structures. This latter loop is positioned between Trp-42 and the active site region and likely plays a role in substrate or peptidyl inhibitor binding. As has been suggested (Tong et al., 1996; Qiu et al., 1996), we speculate that movement in the region of these loops upon binding of inhibitor or substrate contributes, in large part, to the observed changes in emission and CD spectra.

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## SUPPORTING INFORMATION AVAILABLE

A synthetic scheme describing the complete synthesis of compounds **3** and **5** as well as the physical properties (NMR, IR, MS, HRMS) of compounds **1**−**6** mentioned in the paper (13 pages). Ordering information is given on any current masthead page.

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