Single-chain Recombinant Human Cytomegalovirus Protease
ACTIVITY AGAINST ITS NATURAL PROTEIN SUBSTRATE AND FLUOROGENIC PEPTIDE SUBSTRATES*

(Received for publication, May 22, 1995)

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We report here the production of active recombinant single-chain human cytomegalovirus protease in Escherichia coli and development of a continuous assay for this protease. In order to produce the human cytomegalovirus (HCMV) protease for structural studies and accurate kinetic analysis, mutation of alanine 143 at an internal cleavage site was introduced to prevent autoproteolysis. The resulting soluble 29-kDa A143Q protease was purified to homogeneity by hydrophobic interaction and ion-exchange chromatography. The in vivo protein substrate, assembly protein precursor, was also expressed and purified for activity studies. To develop a continuous protease assay, fluorescent synthetic peptide substrates similar to the cleavage sequence P5 to P5 of the maturation site containing anthranilic acid and nitrotyrosine as a resonance energy transfer donor-acceptor pair were designed. Purified HCMV A143Q protease cleaved the recombinant assembly protein precursor with $K_m$ and $k_{cat}$ values of $3.0 \pm 1.0 \mu M$ and $13.3 \pm 1.6 \text{min}^{-1}$. The $K_m$ for peptide substrates is at least 45-fold higher than for the natural protein substrate, but the $k_{cat}$ values are similar. A sensitive assay was developed using fluorescent peptide substrates, which can detect $\mu$M HCMV protease activity.

The Herpesviridae family includes several human pathogenic species such as herpes simplex virus 1 and 2 (HSV-1 and -2),
\(^1\) cytomegalovirus (CMV), Epstein-Barr virus, and varicella-zoster virus. Viral infection by HCMV is very common, and 40–80% of population becomes infected by HCMV before adulthood (1). HCMV is a serious pathogen in immunocompromised individuals, especially those patients with AIDS, receiving organ or bone marrow transplants, or undergoing cancer chemotherapy or steroid therapy. CMV can cause damage in many organs, including the lung, retina, liver, and gastrointestinal tract. Ganciclovir and foscarnet are inhibitors of viral DNA polymerase and have been used to treat HCMV infections; however, they have the undesired side effects of nucleotide analogs (2).

All members of the Herpesviridae family are similar at both the morphological and genomic levels. Herpesviruses contain a DNA genome of over 100 kilobases, an icosahedral capsid, and a lipoprotein envelope. The viral genome replicates inside the nucleus of infected cells and is then packaged into an intermediate capsid and followed by the acquisition of a nuclear membrane envelope and release of the virion from the infected cell. The HSV-1 assembly protein precursor, ICP35, is a major component of the intermediate capsid but is absent in mature virions (3–5). During virion maturation, ICP35 undergoes proteolytic processing to generate the mature assembly protein that lacks approximately 20 amino acids from the carboxyl terminus. An HSV-1 temperature-sensitive mutant (ts1201) that is defective in the processing of ICP35 has been reported by Preston et al. (6). This mutant virus fails to package progeny viral DNA into virions at the nonpermissive temperature, and only empty capsids accumulate. This ts lesion was mapped to the UL26 ORF. The UL26 ORF overlaps with the ICP35 producing UL26.5 ORF, and these two ORFs are 3’-coterminal (6). Liu and Razman (7) reported that the product of the UL26 ORF is an 80-kDa protease that is responsible for the proteolytic processing of ICP35 protein. The UL26.5 promoter lies within the UL26 ORF. Translation initiation of ICP35 begins at methionine codon 307 of the 80-kDa protease. The amino acid sequence of the ICP35 is therefore identical to the carboxy-terminal region of the 80-kDa protease. During viral replication, the 80-kDa protease cleaves both itself and ICP35 at a common maturation site approximately 5 kDa from the shared carboxyl terminus. The 80-kDa protease also undergoes a unique autoproteolytic cleavage at a release site between amino acid residues 246 and 247, resulting in the release of a 29-kDa catalytic domain. These findings, together with the recent work done with the UL26 null mutant virus (8) suggested that herpes proteases play a critical role in viral particle maturation and are attractive targets for anti-viral chemotherapy.

The human CMV UL80 ORF was reported to share homology with HSV-1 UL26 ORF (9) as well as with the assembly protein nested genes ORF of the simian cytomegalovirus (10, 11). Gibson and co-workers (12, 13) have demonstrated that the carboxy-terminal region of the HCMV UL80 encodes a protein that is proteolytically processed in a similar fashion as the HSV-1 ICP35. By transient transfection assays, the simian CMV assembly protein nested gene-1 ORF was shown to encode a 590-amino acid protease whose amino-terminal 249 residues possessed proteolytic activity. This region also contains sequence motifs that are highly homologous to proteases of other herpesviruses (14). More recently, Baum et al. (15) used \(^35\)S methionine labeling of Escherichia coli harboring expression plasmids containing UL80 ORF sequence and found an 85-kDa protease that underwent autoproteolysis in vivo and produced a set of proteins with molecular masses of 50, 29, 16, 13, and 5 kDa (15). The three cleavage sites of the 85-kDa
protease were identified as follows: (i) a maturation (M-) site at the carboxyl terminus, which shares sequence identity with the cleavage site of assembly protein precursor, (ii) a release (R-) site, which yielded the 29-kDa amino-terminal catalytic domain and a 50-kDa carboxyl-terminal fragment, and (iii) an internal (I-) site that lies within the 29-kDa catalytic domain between Ala-143 and Ala-144.

The design of inhibitors for the HCMV protease requires a detailed understanding of its structure and catalytic mechanism. The major difficulty in obtaining pure and stable enzyme for such studies has been due to autoproteolysis, which results in cleavage within the catalytic domain. We have expressed the stable single-chain active HCMV A143Q protease, purified it to homogeneity for crystallographic studies, and developed a sensitive continuous protease assay using fluorogenic peptides as substrates for the kinetic studies.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression of the HCMV Protease Catalytic Domain—DNA isolated from cultured cells infected with HCMV strain AD169 was used as the template for polymerase chain reaction amplification of the coding sequences for both the protease catalytic domain (amino acids 1–256) and assembly protein precursor. Oligonucleotide primers, were synthesized based on the published HCMV UL80 ORF sequences (9). All amplifications were performed with the Taq polymerase, the Perkin-Elmer/Cetus Gene Amp DNA kit, and the standard procedure (16). Amplified DNA fragments were separately digested with Ndel and BglI prior to ligation into corresponding sites of the expression plasmid pMGH4 (17). Substitutions of the Ala-143 within the protease catalytic domain was introduced using Kunkel's site-directed mutagenesis method (18). The resulting constructs, pCMVPI-1 encoding the protease catalytic domain and pPAP-6 encoding the assembly protein precursor, were confirmed by both restriction enzyme mapping and nucleotide sequencing (19). E. coli strain BL21(DE3) (Novagen; Ref. 20) was used as the expression host to produce the recombinant HCMV protease. The bacterial cells harboring expression constructs were cultured in 2 x YT medium (16 g/liter Bacto-tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) at 37°C containing 100 μg/ml ampicillin. Cultures growing to mid-log phase were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. In 2 h after induction, the cells were harvested by ultracentrifugation (Pellicon, Millipore) and centrifugation. Small samples of total lysates were prepared from cultures and subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue (21).

Purification of HCMV A143Q Mutant Protease Catalytic Domain—All purification steps were carried out at 4°C. Approximately 30 g (wet weight) of induced bacterial cell pellet was suspended in 20 ml of lysis buffer (40 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM DTT) and lysed by passing through a microfluidizer (Microfluidics Corp.). The lysate was centrifuged at 35,000 rpm for 50 min in Ti 45 rotor at 4°C to pellet any insoluble material. After adding NaCl to a final concentration of 2.75 M, the soluble lysate was loaded on a phenyl-Sepharose hydrophobic interaction column, and eluted with a 2.75–0 M NaCl gradient in 20 ml Tris-HCl, pH 8.0, 1 mM DTT. Fractions containing the A143Q protease were pooled and dialyzed against 20 ml Tris-HCl, pH 8.0, 1 mM DTT. The dialyzed pool was then loaded on a 75-ml Q-Sepharose column (Pharmacia Biotech Inc.), which was equilibrated in DT buffer (1 mM DTT) and 20 ml Tris-HCl, pH 8.0), followed by a 180 ml of DT buffer washed 3-4 ml with a 675 ml of linear gradient of 0–300 mM NaCl in DT buffer. Fractions containing HCMV protease were pooled, dialyzed against DM buffer (1 mM DTT, 20 mM Mes, pH 6.0) and loaded onto a 75-ml S-Sepharose column (Pharmacia) equilibrated with the same buffer. The column was washed with 120 ml of DM buffer and eluted with a 675-ml linear gradient of 0–50 mM NaCl in DM buffer. Fractions containing the A143Q protease were then concentrated to approximately 150 mg of the purified protease. The catalytic domain sequence of purified HCMV protease was determined after separation by SDS-PAGE and electroblotting onto Immobilon-P transfer membrane (Millipore). Automated Edman degradation was carried out on an Applied Biosystems model 477A pulse-liquid sequencer (22). The free thiol groups of A143Q protease were determined by Ellman titration (23). The A143Q protease was dissolved in 50 mM phosphate buffer, pH 7.3. Each 1 ml sample containing 2 nmol of purified A143Q protease in 0.1 M phosphate buffer, pH 7.3, 1 mM EDTA, with or without denaturation in 6 M guanidinium chloride, was incubated with 50 μl of 3 mM 5,5'-dithiobisnitrobenzoic acid at room temperature. The release of nitrothiobenzoate was monitored by the increase in absorbance at 412 nm from which the molar concentration of free thios was calculated based on the molar extinction coefficients of nitrothiobenzoate at 412 nm being 13,700 and 14,150 M⁻¹ cm⁻¹, respectively, in the presence or absence of 6 M guanidinium chloride.

Purification of HCMV Assembly Protein Precursor—The soluble lysate was prepared from induced bacterial culture as described above except for the addition of the following protease inhibitors: 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.6 g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The soluble fraction of the E. coli lysate was filtered through a 0.45-μm spin filter and loaded onto a 20-ml Q-Sepharose equilibrated to the same buffer. Flow-through fractions were collected, dialyzed against PD buffer (1 mM sodium phosphate buffer, pH 6.9, 1 mM DTT), and loaded onto a 20-ml hydroxyapatite column (Bio-Rad). The column was washed with 60 ml of PD buffer and eluted with a 300-ml linear gradient of 0–500 mM MgCl₂ in PD buffer. Fractions containing the assembly protein precursor were pooled and concentrated to at least 0.65 mg/ml by ultrafiltration using a 10-kDa cut-off polysulfone membrane (Millipore). Ammonium sulfate was added to the concentrated protein solution to a final concentration of 1.62 M and centrifuged at 30,000 × g for 30 min. The protein pellet was resuspended in one-fourth of the initial volume and precipitated by adding potassium chloride to 1.7 M and centrifugation. The final protein which contained assembly protein precursor, was resuspended in 10 mM HEPES, pH 7.0, 1 mM DTT.

Natural Protein Substrate Cleavage Assays—Purified assembly protein precursor was incubated at 37°C with purified HCMV A143Q protease in 20 mM HEPES, pH 7.0, and 6 mM DTT without additional co-solvents. After incubation, sample mixtures were boiled immediately and subjected to SDS-PAGE. After Coomassie Blue staining, intensities of both 45-kDa assembly protein precursor and the 40-kDa cleaved assembly protein were determined by densitometric tracing using a Molecular Dynamics personal densitometer.

**Determination of K_m and k_cat for the Natural Protein Substrate by Saturation Analysis**—A143Q protease at 58 ng and assembly protein substrate precursor at 1.08–8.6 μg were incubated as described above, and samples were taken at 0, 2, 4, 6, and 8 min. Each sample contained 100 ng (3.5 pmol) of A143Q protease and 2.5, 5, 10, or 20 μg (65–520 pmol) of assembly protein precursor. These samples were subjected to SDS-PAGE and Coomassie Blue staining as described above. Potential nonlinearity in densitometric tracing was eliminated by electrophoresing amounts equivalent of 2.5 μg of substrate from all samples. Known amounts of protein were also electrophoresed with each series of samples as a control for densitometric tracing. The percentage of remaining uncleaved assembly protein precursor was calculated from the densitometric intensity and plotted against time for each substrate concentration. Data from each substrate concentration were fit to a single exponential decay curve using KaleidaGraph data analysis software (Abelbeck Software). The time required for 10% substrate cleavage was calculated from the fitted curves, and reaction rates were then calculated as pmol of substrate cleaved per second. The K_m and k_cat values were determined by plotting the reaction rates versus substrate concentration. Data from each substrate concentration were fit to a single exponential curve using KaleidaGraph data analysis software (Abelbeck Software). The time required for 10% substrate cleavage was calculated from the fitted curves, and reaction rates were then calculated as pmol of substrate cleaved per second. The K_m and k_cat values were determined by plotting the reaction rates versus substrate concentration. Data from each substrate concentration were fit to a single exponential decay curve using KaleidaGraph data analysis software (Abelbeck Software).

**HPLC-Based Peptide Assay**—The peptide A6376 (Table I) was synthesized by American Peptide Co., Sunnyvale, CA. The standard 100-μl cleavage reaction contained 25 mM MOPS, pH 7.2, 50% glycerol, 1 mM DTT, 0.05% SDS, 200 μM A6376, 0.1 M phosphate buffer, pH 7.3, 1 mM EDTA, with or without denaturation.
tion while less than 5% of the substrate was cleaved. The reaction was
stopped by adding glacial acetic acid to a final concentration of 2%. The
reaction mixture was then subjected to reverse phase HPLC using a
POROS R2H column (Perceptive Biosystems). Products generated
by cleavage were resolved from substrate, and other components
with a 1–12% acetonitrile gradient in 0.1% trifluoroacetic acid/H2O.
Product peak areas were determined and used to calculate initial rates.

Continuous RET Fluorogenic Assay—PK03 and AM1013 (Table I)
are internally quenched fluorogenic peptide substrates that were
synthesized by Enzyme Products Systems, Dublin, CA, and American
Peptide Co., respectively. The anthranilic acid group emits fluorescence
at 420 nm when excited with 320 nm monochromatic light. The fluo-
rescence of the anthranilic acid group is significantly quenched by
resonance energy transfer to the nitrotyrosine residue (24). When the
peptide is cleaved at the A/S scissile bond, an increase in the observed
fluorescence of the anthranilic acid is obtained. The standard 600-nM
cleavage reaction was performed at 25 °C in a microfluorescence cuvette
containing 25 mM MOPS, pH 7.2, 50% glycerol, 1 mM DTT, 2% Me2SO
(same as for HPLC assay), and 2 μM peptide substrate. The concen-
tration of HCMV protease was 80 nM so that less than 5% of the
substrate was cleaved over the 5-min reaction time. The initial rates
were calculated as the increase in fluorescence versus time.

Determination of $k_{\text{cat}}$, $K_m$ for Peptide Substrates by Progress Curve Analysis—For the fluorogenic assay, the concentrations of HCMV protease, PK03, and AM1013, were 0.5–1.0, 20, and 20 μM, respectively. The continuous change in fluorescence was monitored. For the HPLC-based assay, the enzyme concentration varied at 1–4 μM, and both the substrate peptide A6376 and product peptide peak areas were monitored. Progress curves were allowed to proceed to completion and were fit to an equation describing a first order single exponential decay using KaleidaGraph. $K_m$ was calculated by $k_{\text{cat}}K_m = k_{\text{cat}}(\text{HCMV protease})$.

Determination of $k_{\text{cat}}$, $K_m$ for Peptide Substrates by Saturation Analysis—The individual values of $k_{\text{cat}}$ and $K_m$ were determined by measuring initial rates with variable substrate concentrations. The initial rates were fitted to the nonlinear form of the Michaelis-Menten equation using KaleidaGraph. The fluorogenic assay could not be used for this experiment since high concentrations of substrate caused inner filter effects. This effect erroneously decreases the apparent rate. Reverse-phase HPLC was therefore used to analyze product formation from PK03 and A6376 after being cleaved by the HCMV protease.

RESULTS AND DISCUSSION

Initially, the expression of the wild-type HCMV protease was
attempted with the T7 expression phagemid pMGH4 (17) in the
E. coli strain BL21(DE3). By using this expression system,
wild-type HCMV protease could only be accumulated as an
insoluble intact 29-kDa protein (Fig. 1A) at 42 °C. Upon refold-
ing, proteolytic degradation of the 29-kDa protease into 16- and
13-kDa fragments started to occur as the protease concentra-
tion was increased to 2 mg/ml (data not shown). The same 16-
and 13-kDa proteins were observed in induced E. coli lysates
prepared from 37°C cultures. These degradation fragments
were previously seen in metabolically labeled E. coli lysates
by Baum et al. (15), and they were identified as the products of a
cleavage event occurring between Ala-143 and Ala-144 at the
I-site. Other groups have recently reported expression and
purification of the wild-type HCMV protease catalytic domain
from inclusion bodies (25, 26) or as a two-chain protease
due to autoproteolytic processing at the I-site (27). As a conse-
quence of this autoproteolytic breakdown, preparation of active
single-chain wild-type HCMV protease at high concentrations
required for crystallographic studies is not feasible.

Ala-143 of the I-site lies between the conserved sequence motifs CD1 and CD2 among herpes proteins. Deletion of the corresponding region in the simian CMV resulted in a stable protease and autolytic cleavage at the I-site was not observed. This mutated protease could still cleave its own assembly protein precursor at the M-site with approximately the same effi-
ciency as the wild-type enzyme (28). In light of this observation,
we mutated the alanine 143 residue of the HCMV protease to
Asn, Ser, Thr, and Val were made. Based on
their autoproteolytic activities in crude lysates (see Fig. 1B),
mutants can be divided into two groups. Group I mutants,
A143G and A143S, still produced 16- and 13-kDa fragments.
Autoproteolytic activities observed with the group I mutants
suggests that the I-site prefers amino acid residues with small
side chains at P1 position. All group II mutants: A143Q,
A143N, A143V, and A143T, produced intact 29-kDa protease
abundantly at 37 °C in E. coli. During the preparation of this
manuscript, a similar approach was reported by Holwerda et
al. (27). They disrupted the I-site by changing valine 141 to
alanine (VEA △ A to AEA △ A) and recovered active 30-kDa
one-chain enzyme. In our expression construct, the histidine
tag used by Holwerda et al. (27) for purification purposes was
avoided so as to obtain crystallographic information reflective
of the native HCMV protease without unnecessary changes.

We chose to purify the HCMV A143Q protease and to test
whether this mutant protease retains proteolytic activity. Purification included phenol-Sepharose, Q- and S-Sepharose chromatographic steps (Fig. 2). HCMV A143Q protease was stable during purification and at 4°C for up to 1 month at less than 10 mg/ml. The amino-terminal amino acid sequencing of purified A143Q showed the correct sequence of HCMV protease with 80% of the protease retaining methionine as the first residue, while the remaining 20% had the initiation methionine cleaved off and thus started at the second residue threonine. Ellman titration showed that only three of the five sulfydryl groups in the native A143Q protease were reactive against 5,5'-dithiobisnitrobenzoic acid. After denaturation, the number of sulfydryl groups/protease molecule accessible to 5,5'-dithiobisnitrobenzoic acid reaction increased from 3 to 4.5. These results indicate that no disulfide bonds exist in the HCMV protease catalytic domain and that two of the five cysteines may be buried in the hydrophobic core of the native protein. The reported thiol content of wild-type HCMV protease after exposure to 2% SDS is 4.9 ± 0.5 (26), which is similar to the 4.5 thiol groups found in the denatured A143Q protease. It was therefore concluded that as an intracellular enzyme, all five cysteines in the catalytic domain of HCMV protease are kept in the reduced form. HCMV protease, unlike papain, does not have cysteine as an active site residue. However, it was observed by us and also reported by others that the HCMV A143Q protease loses activity rapidly in the absence of DTT (26) and that DTT prevented the loss of activity. It is clear that the cysteine residues need to be reduced for maximal activity. Therefore, during experiments of extended duration, DTT was supplied exogenously at 1-10 mM. However, for experiments with short incubation time, the DTT at 8-12 µM carried over from the enzyme stock was adequate.

The same expression phagemid pMGH4 was used to express the HCMV assembly protein precursor in E. coli. The recombinant assembly protein precursor was soluble in the lysate. Purification included Q-Sepharose, hydroxylapatite column chromatography, and salt precipitations, and yielded assembly protein precursor with sufficient purity for enzymatic studies. During purification, it was observed that assembly protein precursor requires 5-10 mM DTT to maintain its solubility. Amino-terminal sequencing confirmed the 45-kDa assembly protein precursor identity and showed that its initiation methionine was removed. The enzymatic activity of purified A143Q was initially demonstrated using the assembly protein precursor. As shown in Fig. 3, 2.5 µg (1.08 µM) of substrate could be completely processed within 10 min by 0.5 µg (0.29 µM) of purified A143Q protease. In the assay buffer, 6 mM DTT was included to optimize the A143Q protease activity and to keep the assembly protein precursor soluble. Cleavage of the assembly protein precursor by the A143Q protease was quantitated by densitometric tracing of the full-length 45-kDa and processed 40-kDa protein bands on Coomassie Blue-stained gels after SDS-PAGE electrophoresis. The $K_m$ and $k_{cat}$ were determined to be 3.0 ± 1.0 µM and 13.3 ± 1.6 min⁻¹, respectively. The large deviation in $K_m$ was mostly due to the experimental limit inherent to this discontinuous gel-based assay. The concentration range of substrate was also limited since the $K_m$ was so low. In vitro proteolytic processing of the HCMV assembly protease made at the M-site by a recombinant mutant V141A single-chain and wild-type two-chain HCMV protease was also recently reported by Holwerda et al. (27). The processing of protein substrate was, however, only about 50% complete in the presence of 20% glycerol and 10 mM DTT after a 19-h incubation. The authors suggest that the incomplete turnover is due to a time-dependent loss of protease activity. The HCMV A143Q is significantly more stable than the mutant V141A and wild-type enzyme as observed both under assay conditions and during storage.

In order to develop a sensitive continuous peptide-based assay for the HCMV protease, three peptide substrates (Table I) mimicking the maturation cleavage site of the full-length HCMV protease were made. Various synthetic peptides were previously used by other groups to measure the HCMV protease activity (25-29). The sequence of the peptide substrate A6376 was the same as the one used by Burck et al. (26). This peptide sequence differs from the native M-site sequence at P6, P2', and P6' positions. These changes were maintained in A6376 for its ease of synthesis and higher solubility as compared with its native sequence. After cleavage of A6376 peptide by the HCMV protease, the amino-terminal peptide product was readily resolved by reverse-phase HPLC. Cleavage at the A/S scissile bond within the A6376 peptide was confirmed by the same retention time observed by HPLC analysis of the synthetic peptide containing only P6 to P1 residues. The terminal sequences of the A6376 peptide were also modified to produce the resonance energy transfer fluorogenic peptides.
In order to establish that the mutant HCMV A143Q protease has the same activity as the wild-type enzyme, a comparison of the enzymatic activity of the HCMV A143Q mutant protease was made against the activities previously reported by various other groups (25–29) for the wild-type counterpart (Table III). This analysis clearly demonstrates that mutant A143Q protease has very similar activity against peptide substrate when compared with the wild-type HCMV protease.

For these substrates and assembly protein substrate, we have not compared the activity of the mutant A143Q and wild-type protease against the natural protein substrate described in the previous section, it can be inferred that the mutant A143Q protease has the same activity as the wild-type enzyme, a comparison of the enzymatic activity of the HCMV A143Q mutant protease was made against the activities previously reported by various other groups (25–29) for the wild-type counterpart (Table III). This analysis clearly demonstrates that mutant A143Q protease has very similar activity against peptide substrate when compared with the wild-type HCMV protease.

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Much factors affected the activity of this protease. Both the Km and Km were affected by changes in the glycerol, Me₂SO, and buffer concentrations. Increasing concentrations of glycerol enhance the formation of peptide cleavage products by 20-fold (Fig. 4). Moreover, this increase in activity can be even greater than the observed 20-fold enhancement since the amounts of product turned over at higher concentrations of glycerol do not represent initial rates of product formation. In the absence of glycerol, increasing concentrations of Me₂SO in the reaction mixture up to 25% enhance the activity (Fig. 5). However, in the presence of 50% glycerol the addition of Me₂SO is harmful to activity. An increase in the concentration of NaCl also decreases the A143Q protease activity. This NaCl-inhibi-

### Table II

| Substrate                        | $k_{cat}^{a}$ | $K_m^{a}$ | $k_{cat}/K_m^{a}$ |
|---------------------------------|--------------|-----------|------------------|
| Assembly protein precursor      | 13.3 ± 1.6   | 3.0 ± 1.0 | 4.4 ± 1.5 (n = 1) |
| A6376                           | 18.5         | 476       | 0.038 ± 0.006 (n = 4) |
| PK03                            | 22.1         | 134       | 0.165 ± 0.003 (n = 5) |
| AM1013                          | ND           | ND        | 0.19 ± 0.01 (n = 6) |

| Substrate                        | $k_{cat}^{b}$ | $K_m^{b}$ | $k_{cat}/K_m^{b}$ |
|---------------------------------|--------------|-----------|------------------|
| Assembly protein precursor      |             |           |                  |
| A6376                           |             |           |                  |
| PK03                            |             |           |                  |
| AM1013                          |             |           |                  |

*At 10 mM HEPES, pH 7.0, 6 mM DTT, 37°C.*

*At 25 mM MOPS, pH 7.2, 50% glycerol, 1 mM DTT, 2% Me₂SO, 25°C.*

*Obtained by saturation analysis.*

*Obtained by progress curve analysis.*

*Calculated using $K_m$ from saturation analysis and the $k_{cat}/K_m$ from progress curve analysis.*

*ND, not determined.*

PK03 and AM1013. The carboxyl-terminal glycine in PK03 and lysine in AM1013 was chosen solely for synthetic reasons. We chose anthranilic acid and nitrotyrosine as the donor and acceptor pair mainly because they showed the sufficient quenching efficiency needed for long range resonance energy transfer over 10–16 amino acids (24). With 10 amino acids corresponding to roughly 30 Å distance between the two chromophores, only 25% of the initial fluorescence (75% quenching efficiency) was observed with PK03 and AM1013. After cleavage of these fluorogenic peptide substrates, the fluorescence of the anthranilic acid moiety increases roughly 4-fold. Part of the starting fluorescence is due to free anthranilic acid contaminating the peptide sample (data not shown). However, the incomplete quenching of the anthranilic acid by the nitrotyrosine is compensated for by the very high quantum yield of anthranilic acid. Reliable initial rates were generated by turning over as little as 100 nM, or equivalent to 0.5% of the fluorogenic substrate during the reaction. There is no measurable rate of substrate hydrolysis in the absence of HCMV protease. The values of $k_{cat}/K_m$ for these substrates and assembly protein precursor are shown in Table I. The fluorescent peptides have larger $k_{cat}/K_m$ values than the A6376 peptide presumably due to the removal of the $K_m$-increasing arginine at the amino terminus rather than the addition of the nitrotyrosine and anthranilic acid moieties. This presumption is supported by AM1013 where the positions of the two moieties are switched with little effect on $k_{cat}/K_m$. From this data, we see that PK03 is a better substrate than A6376 by a factor of 4.3-fold. This continuous fluorescent assay is very sensitive and able to detect potential subtle differences between the two enzymes. In the previous section, it can be inferred that the mutant A143Q protease has the same activity as the wild-type enzyme, a comparison of the enzymatic activity of the HCMV A143Q mutant protease was made against the activities previously reported by various other groups (25–29) for the wild-type counterpart (Table III). This analysis clearly demonstrates that mutant A143Q protease has very similar activity against peptide substrate when compared with the wild-type HCMV protease.

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A143Q protease can recognize and cleave its protein precursor efficiently and is sufficient in cleaving the assembly from amino acid substitution, the A143Q mutant can be purified from E. coli as a stable single-chain 29-kDa protein, (ii) purified A143Q protease can recognize and cleave its in vivo protein substrate efficiently and is sufficient in cleaving the assembly protein precursor in vitro without other cellular or viral factors, (iii) a sensitive, continuous peptide-based fluorescent assay has been developed for the HCMV protease to detect activity in nM concentration of enzyme (Fig. 7), and activity detected with peptide substrates has $k_{\text{cat}}$ values similar to that measured with the protein substrate, and (iv) A143Q mutant enzyme has the same, or similar, activity as the wild-type enzyme.

The HCMV protease has recently been demonstrated by Holwerda et al. (27) to be a serine protease, and Ser-132 was identified as the active site nucleophile based on the irreversible modification by the serine protease inhibitor diisopropyl fluorophosphate. Similar irreversible modification of the HSV-1 protease at Ser-129 by diisopropyl fluorophosphate was reported by Dilanni et al. (29). And, mutation of the equivalent Ser residue in the simian CMV protease to Ala also eliminated the enzyme activity (28). Since HSV and CMV are members of the Herpesviridae family, and their proteases share sequence homology, it suggests that both HCMV and HSV-1 protease could belong to the serine protease class. Inhibition of the HCMV protease by E. coli N-ethylmaleimide and iodoacetamide, at high inhibitor/enzyme molar ratio, was also documented (26) and confirmed in this laboratory (data not shown). However, this inhibition may be due to nonspecific alkylation of Cys residues leading to inactivation of the enzyme.

Interestingly, the recently determined human rhinovirus 3C...
1.1 The time course was collected at 25°C in 25 mM MOPS, pH 7.2, 8 µM DTT, 50% glycerol, and 4% MeSO. The concentration range of PK03 was 0.02-400 µM and 100-2000 µM; the concentration of HCMV protease was 0.164 and 1.14 µM for the 10 mM MOPS and 100 mM MOPS datasets, respectively. For clarity, only 5% of the collected data points are shown. The data were fit to a single exponential equation with the amount of product formed per minute during a 15-min incubation.

The data were shown as a double-reciprocal plot where $V_0$ represents the activity of PK03 and HCMV A143Q protease was measured by the HPLC assay at 25°C for 15 min in 10 or 100 mM MOPS, pH 7.2, 88-95.

The dataareshownasadouble-reciprocalplotwherestarstherepresentationoftheHCMVproteaseactivitymeasuredbytheHPLCassayat25°Cfor15minin10ormMOPS,pH7.2,8µMDDT,50%glycerol,and4%MeSO.ThecatalyticresiduesCys-146,His-40,andGlu-71havetotal-16and1.14µMforthe10mMand100mMdata sets,respectively.

2.2 The different spacing of the HCMV protease active site residues is highlighted.

3.3 The geometric arrangement within the catalytic triad of this new class of viral proteases has been determined.

4.4 The value of $K_{cat}/K_m$ for A6376 is shown.

5.5 The concentration of HCMV protease was 0.164 and 1.14 µM for the 10 mM MOPS and 100 mM MOPS datasets, respectively.

6.6 The concentration of PK03 and HCMV A143Q protease were 20 and 0.67 µM, respectively. For clarity, only 5% of the collected data points are shown. The data were fit to a single exponential equation with $k_{cat}/K_m$ calculated as described under "Experimental Procedures."

7.7 The protease structure reveals its three-dimensional structure possesses a trypsin-like polypeptide fold, and its catalytic residues Cys-46, His-40, and Glu-71 have an overall geometry similar to that of the Ser-His-Asp catalytic triad found in trypsin-like serine proteases (30). The human hanoi virus protease structure demonstrates that this trypsin-like viral protease has a cysteine in place of serine to serve as an active site nucleophile. For the HCMV protease, His-63 and Glu-122 were recently identified as two other residues in the active site triad (31). The different spacing of the HCMV protease active site residues: His-63, Glu-122, and Ser-132 and the lack of sequence homology with other known serine proteases together suggest that herpes proteases belong to a new class of serine proteases and their structures may be completely different from any solved serine protease structure. The structure solution of the HCMV A143Q protease is currently being pursued. Three-dimensional structure of the HCMV protease will undoubtedly reveal if the Ser-132 is indeed the active site residue and its geometric arrangement within the catalytic triad of this new class of viral protease.

Acknowledgments—We thank Karen Fan for the gift of HCMV-infected culture cells. We also thank Zdenek Hostomsky and Howard Tenenbaum for E. coli cultures grown in the fermenter and Anthony Welch for helpful discussions.

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