The quaternary state of the human cytomegalovirus (hCMV) protease has been analyzed in relation to its catalysis of peptide hydrolysis. Based on results obtained from steady state kinetics, size exclusion chromatography, and velocity sedimentation, the hCMV protease exists in a monomer-dimer equilibrium. Dimerization of the protease is enhanced by the presence of glycerol and high concentrations of enzyme. Isolation of monomeric and dimeric species eluted from a size exclusion column, followed by immediate assay, identifies the dimer as the active species. Activity measurements conducted with a range of enzyme concentrations are also consistent with a kinetic model in which only the dimeric hCMV protease is active. Using this model, the dissociation constant of the protease is 6.6 μM in 10% glycerol and 0.55 μM in 20% glycerol at 30 °C and pH 7.5.

Viruses of the herpes family, including the human cytomegalovirus (hCMV) and herpes simplex virus, encode a protease essential for viral capsid formation and viral replication (1-3). The herpesvirus proteases are synthesized as precursor proteins that undergo autoproteolytic processing during viral assembly. One of the natural substrates is the viral assembly proteins involved in the construction of intermediate viral capsids within the infected cell nucleus. The other natural substrate is the protease precursor protein. The protease catalytic domain is localized in the N terminus of the precursor, which in the case of hCMV encompasses the N-terminal 256 amino acids of the 708-amino acid precursor protein (4, 5).

It has been suggested that the hCMV protease is a serine protease based on its chemical reactivity toward classical serine protease inhibitors (6), and recent site-directed mutagenesis data (7) have implicated the catalytic triad of hCMV protease to be Ser-132, His-63, and Glu-122. However, the catalytic efficiency of the hCMV protease is orders of magnitude less than that expected of a classical serine protease, and no amino acid sequence homology has been found between this enzyme and the well characterized serine proteases (7, 8).

This report identifies the dimerization of mature hCMV protease as a critical property governing its catalytic activity. Our data are consistent with a dimer dissociation constant (Kd) in the low micromolar range with the dimeric protease being the active species. Given the proposed identity of the catalytic triad and the classification of this enzyme as a serine protease, our findings mark activation by dimerization as a unique feature of this new member of the serine protease group.

MATERIALS AND METHODS

Enzyme Expression and Purification—Both the wild-type and a mutant form (V141G and V207G mutations) of the hCMV protease were expressed in Escherichia coli as described previously (6, 7). The resulting hCMV protease resistant to autoproteolysis. All purification steps were performed at 0-4 °C. For the mutant, lysis of cells was performed with a microfluidizer (Microfluidics Corp., Newton, MA) in 50 mM Tris-HCl, pH 8.0 buffer containing 10% glycerol (v/v throughout), 25 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. The lysate was centrifuged, and the pellet was washed with lysis buffer plus 0.1% Nonidet P-40 and recentrifuged. Inclusion bodies were dissolved with 7 M urea, 50 mM Tris-HCl, 5 mM DTT, pH 8.0, followed by centrifugation and chromatography on a MonoQ column (Pharmacia Biotech Inc.). Elution was performed using a sodium chloride gradient in the urea-containing buffer. Protein folding was accomplished by dilution of protease-containing fractions to 0.2 mg/ml into 25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 5 mM DTT, and 1 mM guanidine HCl, followed by dialysis in the same buffer without guanidine HCl for 24 h. The resulting protein solution was chromatographed on a MonoQ column and eluted with a sodium chloride gradient in 25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, and 1 mM DTT to yield the purified enzyme. The wild-type protease was purified similarly. To avoid self-proteolysis, the protein obtained from the first MonoQ step was folded and dialyzed as above, but in the absence of glycerol. The sample was then acidified to pH 5.5 with MES, applied to a Merck Fractogel SO4 column in 50 mM MES, 1 mM EDTA, 1 mM DTT, pH 5.5, and eluted with a sodium chloride gradient. The wild-type enzyme was stable at pH 5.5 and returned to full activity following dilution to pH 7.5. Enzyme preparations were greater than 95% pure by SDS-PAGE and gave the expected amino acid analysis. The N-terminal 5 residues of both enzymes were MTMDE, showing retention but deformylation of the initial N-formyl methionine. Electro spray mass spectrometry indicated a single species within 10 atomic mass units of the expected mass. The concentrations of stock enzyme solutions were determined by quantitative amino acid analysis. The data presented here were collected with use of the stable V141G/V207G mutant enzyme. The wild-type enzyme was employed to confirm all kinetic phenomena as characteristic of the hCMV protease.

Kinetic Assays and Equilibrium Constants—Peptide substrates used were either the fluorophore-labeled (Daboy)-RGVVNASRLA-(Edans) (Bachem Biosciences, Philadelphia, PA) or Ac-RGGVNASRLA-bu.RLATR-amide (Midwest Biotech, Indianapolis, IN). Products were quantified on high pressure liquid chromatography with fluorescence monitoring of the SSRLA-(Edans) product (350 nm excitation, 500 nm emission) or the Ac-RGWGVNA product (280 nm excitation, 350 nm emission). Reactions were performed for 1 min in a pH 7.5 buffer containing 52 mM MES, 52 mM TAPSO and 100 mM diethanolamine, 1 mM EDTA, 1 mM DTT, 0.05% BSA, bovine serum albumin; MES, 4-morpholinobenzenesulfonic acid; TAPSO, 3-(N-tris(hydroxymethyl)-methylamino)-2-hydroxypropanesulfonic acid.

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1 The abbreviations used are: hCMV, human cytomegalovirus; Abu, L-α-amino butyric acid; Daboy, 4-(4-dimethylaminoophenazono)benzoic acid; Edans, 5-(2'-aminoethyl)-amino-naphthalene-1-sulfonic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MES, 4-morpholinobenzenesulfonic acid; TAPSO, 3-(N-tris(hydroxymethyl)-methylamino)-2-hydroxypropanesulfonic acid.

2 V. Sardana, personal communication.
Km determination, substrate concentrations during assay were 92 μM (Dab,yl)-RGVNVASSRLA-(Edans) or 87 μM Ac-RGWGVNAS,Abu.R-LATR-amide. The apparent dissociation constant (Kd) for monomer-dimer equilibrium, wherein only the dimer form of the enzyme contributes to the observed velocity, was calculated with the equation

$$v_{max} = \frac{v_d}{[E] + [M]}$$  \tag{1}

where v_d is the velocity for dimers, [E] is the total concentration of enzyme (in monomer equivalents), and [M] is the concentration of monomers. The value of [M] is given by

$$[M] = \frac{2 \cdot [E]}{2 + \left( \frac{K_d}{2} + \left( \frac{K^2}{4} + 2K_d \cdot [E] \right)^{0.5} \right)}$$  \tag{2}

and is derived from the equilibrium condition.

\[ K_d = \frac{2 \cdot [M]^2}{[E] - [M]} \]  \tag{3}

**RESULTS**

Dependence of Protease Specific Activity on Enzyme and Glycerol Concentrations—Dilution of a concentrated solution of hCMV protease produces a time-dependent change in activity. At 30°C, the decrease in protease activity occurs within minutes, while at 0°C, no significant change is observable for a period of hours. The change in hCMV protease activity at four temperatures upon dilution as a function of time is shown in Fig. 1. At temperatures above 20°C, the change in activity upon enzyme dilution is rapid enough (t_v ≤ 30 min) that an accurate assessment of activity requires a short assay (~1 min).

The specific activity of the hCMV protease increases at higher enzyme and glycerol concentrations. These effects at 30°C are shown in Fig. 2. The specific activities of the protease measured after enzyme dilution and incubations of 1.5 and 3.5 h prior to reaction are shown in Fig. 2A. The data reveal that the hCMV protease specific activity tends toward zero as its concentration is lowered. The negligible difference between the determinations at 1.5 and 3.5 h suggests that the active form of the enzyme has reached equilibrium within 1.5 h, for both the 10% and 20% glycerol samples. The activity of the protease incubated in 10% glycerol (v/v) is lower than that incubated in 20% glycerol (v/v). It can be shifted back to the higher activity seen for 20% glycerol by addition of an equal volume of buffer containing 30% glycerol to produce a solution containing 20% glycerol, followed by further incubation. Thus, the dependence of hCMV protease activity on enzyme and glycerol concentrations is reversible. The reversal effect is shown in Fig. 2B.

The kinetic parameters Vmax and Km for the substrate (Dab,yl)-RGVNVASSRLA-(Edans) were determined with different hCMV protease concentrations preincubated in 20% glycerol using assay conditions as in Fig. 2. The Vmax and Km values obtained with 0.5 μM enzyme are 360 nmol min⁻¹ mg⁻¹ and 92 μM, respectively, while for 2.0 μM enzyme they are 600 nmol min⁻¹ mg⁻¹ and 97 μM. Thus it is the apparent turnover rate of the enzyme that varies with enzyme concentration.

Analytical Centrifugation—The quaternary state of the hCMV protease was characterized by sedimentation velocity measurements. Table I shows the results of sedimentation analyses of samples at a concentration of 20 μM in the presence or absence of 20% glycerol (v/v). An approximate solution of the Lamm equation can be fit directly to the data to obtain the sedimentation coefficient, S_{20w}, the diffusion coefficient, D_{20w}, and by use of the Svedberg relation, the molecular weight (11). For both samples good fits are found for a single sedimenting species. In the absence of glycerol the molecular weight obtained is 29.7 × 10^5, which is close to the molecular weight of a monomeric hCMV protease. In 20% glycerol, 48.5 × 10^5 is obtained, indicating that the enzyme is in a predominantly

![Fig. 1. Activity of the hCMV protease as a function of time after dilution at 0, 25, 30, and 37°C. A 2.5 μM hCMV protease sample (monomer equivalents) at 0°C was diluted 50-fold to 50 nM into a buffer (52 mM MES, 52 mM TAPSO, 100 mM diethanolamine, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.05% BSA, pH 7.5) at the temperature indicated, and at the times shown an aliquot was withdrawn and assayed with the substrate Ac-GVNVNAS,Abu.R-LATR-amide at the same temperature. Assays were performed for a period of 1 min as described under “Materials and Methods.”](https://www.jbc.org/content/7446/2/7446/F1)

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obtained before assay initiation. The corresponding to a dimeric protease show no evidence of covalent
below 10 

37 

incubated in the absence of glycerol and applied to a size
stable dimeric protease in 20% glycerol.

shown in Table I are both consistent with the existence of a
dimeric form.3 The increase in

protease concentration of 50 

mg 

protease containing 30% glycerol (v/v) to give a final glycerol concentration of 20% and incubated for an additional 2 h. Assays of these samples were conducted alongside those incubated continuously in 20% glycerol. The data for the 10%-shifted-to-20% glycerol samples (○) are shown. B, samples of the enzyme dilutions incubated at 30 °C for 1.5 h in 10% glycerol were mixed with an equal volume of buffer containing 30% glycerol (v/v) to give a final glycerol concentration of 20% and incubated for an additional 2 h. Assays of these samples were conducted alongside those incubated continuously in 20% glycerol. The enzyme concentrations indicated on the

abscissa
glycerol. The enzyme concentrations indicated on the

ordinate
glycerol. The enzyme concentrations indicated on the

ordinate
glycerol. The enzyme concentrations indicated on the

ordinate

specific solid lines correspond to fits of a monomer-dimer equilibrium relationship (see “Materials and Methods” and “Discussion”) wherein the monomer is inactive. The

Kd 

3

values of 6.2 

μM (1.5 h) and 6.9 

μM (3.5 h) were obtained in 10% glycerol and

Kd 

3

values of 0.58 

μM (1.5 h) and 0.51 

μM (3.5 h) were obtained in 20% glycerol. The 10%-shifted-to-20% fit produced a

Kd 

3

of 0.84 

μM. The average 

v0.5 

or velocities for fully dimerized enzyme under these conditions (see “Materials and Methods”), are 403 nmol min

-1 mg

-1 for 10% glycerol and 400 nmol min

-1 mg

-1 for 20% glycerol.

dimeric form.3 The increase in

S20,w and the decrease in

D20,w shown in Table I are both consistent with the existence of a stable dimeric protease in 20% glycerol.

Size Exclusion Chromatography—The hCMV protease preincubated in the absence of glycerol and applied to a size exclusion column elutes with an apparent molecular weight of 37 

× 10

3 or 56 

× 10

3, depending the loading concentration of the protease. With 20% glycerol present during preincubation, the protease elutes as a single 56 

× 10

3 species except with relatively low protease loading concentrations (<10 

μM), where two fractions emerge with apparent weights of 55 

× 10

3 and 33 

× 10

3, as summarized in Table II. Since the molecular weight of the hCMV protease calculated from amino acid sequence is 28 

× 10

3, these results suggest that the protease exists in a monomer-dimer equilibrium. Eluted enzyme samples corresponding to a dimeric protease show no evidence of covalent (disulfide) linkages as demonstrated by SDS-PAGE under non-reducing conditions. The elution profiles obtained from samples equilibrated in 20% glycerol at 30 °C, analyzed on the BioSelect columns run at 10 °C in 20% glycerol, are shown in Fig. 3A.

Assignment of the early and late elution peaks in Fig. 3A as dimer and monomer, respectively, allows the estimation of a

Kd 

for dimerization. Using the ratios of the areas under-the-peak of the early and late peaks, a 

Kd 

value of 0.54 

μM is found for 20% glycerol as shown in Fig. 3B. The maximum fraction of dimeric protease extrapolated from Fig. 3B is 92%. In separate experiments using protease at a loading concentration of 100 

μM, the maximum fraction of dimeric protease is ~0.95. When activity assays are conducted at 0 °C, no detectable activity is found in the eluted peak corresponding to the monomer while hydrolytic activity (~50-fold of detectable level) is found for the dimer peak. The same analysis applied to enzyme pre-equilibrated in 10% glycerol produces a 

Kd 

of 5.5 

μM. The activity data sets shown in Fig. 2 can now be justifiably treated with a model wherein an inactive monomeric hCMV protease exists in equilibrium with an active dimeric hCMV protease. Fits of this model (see “Materials and Methods”) to the kinetic data (Fig. 2) give average dissociation constants ( 

Kd 

) for hCMV protease of 6.6 

μM in 10% glycerol and 0.55 

μM in 20% glycerol.

Changes in sample loading volume, column temperature, and chromatography time have been made to affirm that equilibrium exchange between protease monomers and dimers is negligible during size exclusion chromatography at 10 °C in 20% glycerol. No significant variation in dimer-monomer peak ratios occurs when injection volumes of 5, 10, 20, or 30 

μl (15–90 pmol) of protease sample are made. Column tempe-
Dimerization of hCMV Protease

FIG. 3. Size exclusion chromatography of the hCMV protease. Samples of hCMV protease in 20% glycerol were maintained at 30 °C for at least 90 min prior to chromatography on two Bio-Rad BioSelect columns (in tandem) at 10 °C, as described under "Materials and Methods." A, elution profiles for samples at concentrations, prior to injection, of 171 nM (a), 355 nM (b), 891 nM (c), 1975 nM (d), 2977 nM (e), and 4501 nM (f). Shown here are the protease fluorescence emission data at 350 nm. Injection volumes were adjusted to give the same total protein injected (30 pmol, monomer equivalents). B, fraction of total protein appearing in the dimer peak (26.2 min) as a function of protein concentration. The solid line corresponds to a fit of the monomer-dimer equilibrium function (see "Materials and Methods") to the data, yielding a $K_d$ of 0.54 μM, with a maximum dimer fraction of 0.92. In separate experiments using a loading concentration of 100 μM protease, the maximum fraction of dimeric protease is ≈ 0.95, suggesting that all the hCMV protease in our purified sample is fully capable of dimerization.

Although only the dimer form of the protease is depicted to bind substrates in Scheme 1, it is not possible for us to state unequivocally that the monomeric form of the enzyme does not bind substrate or does not possess a very low catalytic activity. Also unclear at present is the stoichiometry of substrate binding to the enzyme. Our efforts continue in clarifying these issues.

Scheme 1 further defines the kinetic parameters of the hCMV protease based on the results reported here. Determinations of the $K_d$ can be complicated during substrate turnover by the interchange of enzyme form on the minute time scale (Fig. 1), so a short reaction time is required for accurate estimates. Our kinetic results (Fig. 2) indicate that the affinity of the monomeric protease for itself at 30 °C is weak with a $K_d$ in the low micromolar range: 6.6 and 0.54 μM in the presence of 10% and 20% glycerol, respectively. Essentially the same $K_d$ values, 5.5 and 0.55 μM, respectively, are obtained from size exclusion measurements (Fig. 3B). Kinetic estimates of $K_d$ with the alternate substrate Ac-RWGVVNA-S.Abu.RLATR-amide are in complete agreement with these values (data not shown).

Given Scheme 1 and the rate of activity relaxation at 30 °C shown in Fig. 1, the 1-min assay measurements should approximate the dimer catalytic activity prior to subunit dissociation by dilution and prior to substrate perturbation of the monomer-dimer equilibrium. The $V_{\text{max}}$ measurements then allow calculation of a $k_{\text{cat}}$ for fully dimeric enzyme using the assumption of a single active site per subunit. The $V_{\text{max}}$ values in 20% glycerol of 360 and 600 nmol min$^{-1}$ mg$^{-1}$ for 0.5 and 2.0 μM total enzyme, respectively, combined with the corresponding fractions of enzyme present as dimer calculated from the $K_d$, 0.48 and 0.69, give similar $k_{\text{cat}}$ values of 21.2 and 24.2 min$^{-1}$. The...
velocity of dimeric enzyme, \( v_d \), expected to be found in the enzyme titration of Fig. 2 can be calculated from the average of these \( k_{cat} \) values, 22.7 min\(^{-1}\), taking consideration of substrate concentration used (92 \( \mu \)M) relative to the average \( K_m \) under these conditions (94 \( \mu \)M) to yield \( v_d \) equal to 401 nmol min\(^{-1}\) mg\(^{-1}\). In fact, \( v_d \) from the curve fit of data in Fig. 2 yields 400 nmol min\(^{-1}\) mg\(^{-1}\) in 20% glycerol. The true \( k_{cat}/K_m \) value for this dimeric enzyme with (Dabcyl)-RGVVNASSRLA-(Edans) at 30 °C in 20% glycerol is therefore 4000 M\(^{-1}\) s\(^{-1}\), several times higher than previously reported for this and related substrates (8–10).

In summary, the data presented here reveal that the hCMV protease exists as a dimeric species under conditions that enhance the specific activity of the enzyme, such as high enzyme concentration or the presence of glycerol. The activating effect of glycerol has been noted previously (8, 15), but not the underlying mechanism of activation. Activity of the protease observed in assays containing no glycerol is likely due to a small concentration of dimers in equilibrium with monomers. Although unusual for a serine protease, this mode of activation may provide an appropriate temporal trigger for proteolytic activity during the assembly of hCMV capsids, a process wherein the protein components become highly concentrated.

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