Characterization of the Monomer−Dimer Equilibrium of Human Cytomegalovirus Protease by Kinetic Methods†

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ABSTRACT: Herpesviruses encode a serine protease that is essential for the maturation of infectious virions. This protease has a unique polypeptide backbone fold and contains a novel Ser-His-His catalytic triad. It exists in a monomer−dimer equilibrium in solution, but only the dimer form of the enzyme is catalytically active. The stability of this dimer is affected by the presence of anti-chaotropic agents. Most of the reported K_d values for this dimer (between 0.6 and 6 μM) are inconsistent with the fact that the protease is routinely assayed at 20−50 nM concentrations, as only monomeric species would be expected with such K_d values. We have characterized the monomer−dimer equilibrium of HCMV protease using a new method, which observes the exchange between dimers of the wild-type enzyme and the active-site Ser132Ala mutant in a titration experiment. The K_d of the dimer was determined to be 8 μM and 31 nM in the absence or presence of anti-chaotropic agents (10% glycerol and 0.5 M Na_2SO_4), respectively. Detailed kinetic analysis also showed that, in addition to the 260-fold stabilization of the dimer, the anti-chaotropic agents produced a 7-fold enhancement in the catalytic activity of the dimer.

Herpesviruses are large, double-stranded DNA viruses that infect most species throughout the animal kingdom (*1*). Human herpesviruses include herpes simplex virus type 1 (HSV-1), HSV-2, varicella-zoster virus (VZV), cytomegalovirus (HCMV), Epstein−Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV, also known as HHV8). They are major pathogens in humans and can cause a variety of serious disorders, such as sexually transmitted diseases (HSV-1 and HSV-2 infections), chickenpox and shingles (VZV), pneumonia and retinitis in immunosuppressed and immunocompromised patients (HCMV).

The genomes of all herpesviruses encode a serine protease, and the catalytic activity of this protease is essential for the production of infectious virions (*2*, *3*). The amino acid sequences of the herpesvirus proteases share no detectable homology to other proteins in the sequence database. Crystal structures of the proteases reveal that they belong to a new class of serine proteases, with a unique polypeptide backbone fold and a novel Ser-His-His catalytic triad (Figure 1A) (*4*−*13*). Structural and biophysical studies demonstrate that herpesvirus proteases are induced-fit enzymes, undergoing a large conformational change upon substrate/inhibitor binding (*14*−*17*).

Solution studies of herpesvirus proteases showed that they exist in a monomer−dimer equilibrium, with the dimer being the active species and the monomer having little or no activity (*3*, *18*−*23*). A dimer of the protease is observed in all the crystal structures, yet there appears to be a complete active site in each monomer of the dimer (Figure 1A). The formation of the dimer is mediated by the packing of helix αF in one monomer against helices αF, αB, and αC in the other monomer (Figure 1B). Structural, biophysical, and biochemical studies of HCMV protease carrying mutations in the dimer interface suggest that the formation of the proper dimer may be required to stabilize the oxyanion hole of the enzyme (*24*). Biophysical and mutagenesis studies of KSHV protease indicate a conformational change in the enzyme monomers upon the formation of the dimer (*23*).

The activity of herpesvirus proteases can be enhanced by up to 800-fold in the presence of anti-chaotropic salts such as sulfates, phosphates, and citrates (*3*, *20*, *25*, *26*). It is generally believed that the activating effects of these agents are due to their stabilization of the dimeric species of the protease. This is partly supported by the reduction of the K_d values of the dimer in the presence of these salts. A K_d of about 60 μM is observed based on analytical ultracentrifugation experiments for HCMV protease in a buffer containing no salt, whereas the K_d is lowered to about 6 μM in the presence of 0.5 M Na_2SO_4 and 10% glycerol (*24*, *27*). From kinetic experiments, the K_d for HCMV protease dimer was determined to be 6 and 0.6 μM in the presence of 10 and 20% glycerol, respectively (*18*), although a K_d value of 2 nM was reported in the presence of 25% glycerol (*19*). For HSV-1 protease, K_d values of 1 or 0.2 μM have been reported, in the presence of 20% glycerol and 0.2 or 0.5 M citrate (*20*). In addition to the stabilization of the dimer, a conformational change in the protease in the presence of the anti-chaotropic salts has also been observed (*15*, *25*, *26*).

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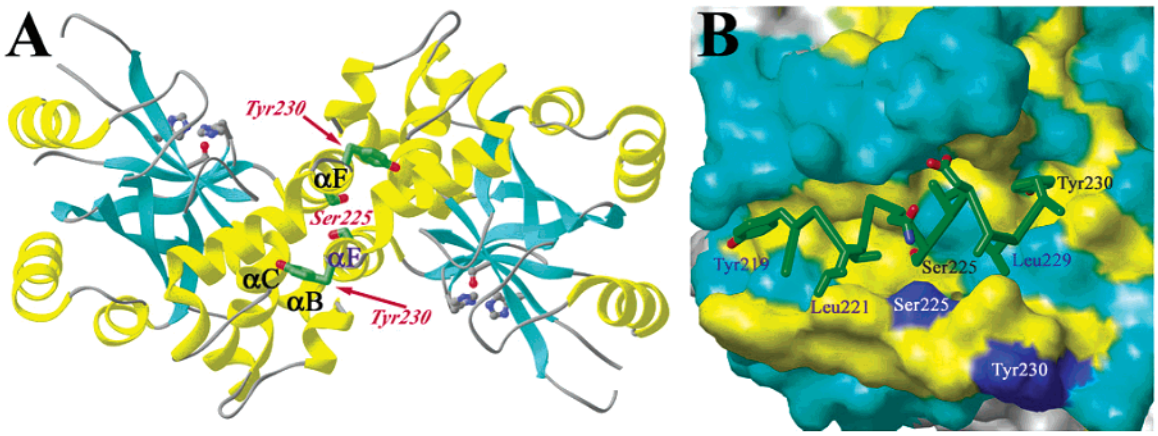

FIGURE 1: Structure of HCMV protease dimer. (A) Schematic drawing of the dimer of HCMV protease. The Ser-His-His catalytic triad, and residues Ser225 and Tyr230 in the dimer interface are shown as stick models. Produced with Ribbons (*32*). (B) The dimer interface of HCMV protease. The molecular surface of residues in the dimer interface is colored in yellow and cyan for hydrophobic and hydrophilic residues, respectively. The Ser225 and Tyr230 residues are colored in blue. Produced with Grasp (*33*).

Kinetic assays for HCMV protease are routinely performed with the enzyme at 20−50 nM concentrations in the presence of anti-chaotropic agents (0.5 M Na_2SO_4 and 10% glycerol). Only monomeric species of the enzyme are expected at such low concentrations, as most of the K_d values reported for the dimer of this protease are between 0.6 and 60 μM. Therefore, it is not clear how the protease can remain active at such a concentration. It was generally believed that the protease dimers dissociated slowly in the assay buffer (from minutes to several hours depending on the buffer condition and temperature) (*3*, *18*), such that during the course of the reaction most of the enzyme remained dimeric even though the concentration was significantly below the K_d.

To obtain a better understanding of the monomer−dimer equilibrium of HCMV protease, we have characterized in more detail the catalytic activity of wild-type and mutants of this enzyme in the "low-salt" buffer (100 mM MOPS (pH 7.5), 2 mM EDTA, 10 mM DTT, and 50 mM NaCl) and the "high-salt" buffer (low-salt buffer supplemented with 0.5 M Na_2SO_4 and 10% glycerol). We show that the wild-type protease at 50 nM concentration in the high-salt buffer can maintain activity for several hours, suggesting that the slow dissociation model is unlikely to be correct. Most importantly, by observing the exchange of monomers between the wild-type and the S132A mutant dimers, we are able to determine the K_d of the dimer in these solutions. The dissociation constant in the high-salt buffer is 31 nM, which can easily explain the observation of catalytic activity of the protease at 20−50 nM concentrations. Moreover, our experiments show that the dimers of the protease dissociate into monomers very quickly in the high-salt buffer, in contrast to expectations from the slow dissociation model.

MATERIALS AND METHODS

Preparation of the Enzymes. The protease samples used in these studies all contain the Ala143Gln(A143Q) mutation, which eliminates the autoproteolysis at this position (*28*). This mutant maintains the catalytic efficiency of the native enzyme, and will therefore be referred to as the wild-type here. The wild-type protease was expressed in *Escherichia coli* strain BL21 (DE3) pLysS, and purified following the protocol described earlier (*4*, *15*). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene), and confirmed by sequencing. All the mutants were expressed and purified using the same protocol as described for the A143Q sample.

Table 1: Summary of Kinetic Parameters

enzyme[a]	buffer	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}M^{-1}$)
wild-type[b]	high-salt	0.16	19.6	8,200
wild-type[c]	high-salt	0.10	11.8	8,500
wild-type + 10 μM S132A mutant[c]	high-salt	0.39	18.8	21,000
wild-type + 67 μM S132A mutant[c]	high-salt	0.39	26.2	15,000
wild-type + 67 μM S132A mutant[c]	low-salt	0.16	55.6	2,900

[a] The wild-type enzyme was at 50 nM concentration, and the AMC substrate was used for all the assays, at 24 °C. [b] Immediately after dilution to the indicated buffer. [c] After overnight incubation in the indicated buffer.

Kinetic Measurements. For the dilution and the titration experiments, the initial velocity for the various protease samples was measured at 24 °C by monitoring the hydrolysis of an internally quenched fluorogenic peptide substrate 4-(4′-dimethylaminophenylazo)benzoyl-Arg-Gly-Val-Val-Asn-Ala↓Ser-Ser-Arg-Leu-Ala((2′-aminoethyl)amino-naphthalene-1)sulfonic acid substrate (Bachem) (λ_{ex} = 355 nm and λ_{em} = 495 nm) (*29*). The activity of the protease was assayed in a low-salt buffer (100 mM MOPS (pH 7.5), 2 mM EDTA, 10 mM DTT, and 50 mM NaCl) and/or a high-salt buffer (low-salt buffer supplemented with 0.5 M Na_2SO_4 and 10% glycerol). The fluorescence signal was monitored using a Photon Technologies Inc. spectrofluorimeter.

The k_{cat} and K_m values for the wild-type and the wild-type/S132A heterodimer in the low-salt and high-salt buffers were obtained at 24 °C using classical Michaelis−Menten kinetics, and extracted using nonlinear least-squares fitting (program Origin, Microcal Software, Inc.) (Table 1). A different substrate was used for these studies, Ac-Cys-Tbg-Tbg-Asn(Me)$_2$-Ala-↓-7-amino-4-methylcoumarin (λ_{ex} = 360 nm, and λ_{em} = 440 nm) (Quantum Biotechnology). This AMC substrate is similar to one reported earlier and exhibits excellent kinetic properties for HCMV protease (*30*). The major advantage of this substrate is that it is not subject to the "inner-filter" effect, so higher concentrations of the

substrate can be used in the assays. This allowed the characterization of the enzyme in the low-salt buffer, which has an elevated K_m value (Table 1). The kinetic parameters for the S225Y/Y230A mutant were obtained also with the AMC substrate. However, as the activity of this mutant is very low, the kinetic parameters were determined using the modified protocol that we described previously (*12*, *24*).

Dilution Experiments. The wild-type enzyme at a fixed concentration, 0.6 or 60 μM in the low-salt buffer, was diluted to a final concentration of 50 nM in the low-salt buffer, and the initial velocity of the enzyme in the presence of 2 μM internally quenched fluorogenic substrate was measured immediately after.

Titration Experiments. The wild-type enzyme at a fixed concentration, 50 nM in the low-salt buffer or 5 nM in the high-salt buffer, was incubated for 24 h with increasing concentrations of the S132A mutant. The initial velocity of the incubation mixtures for the hydrolysis of the internally quenched fluorogenic substrate was determined at 24 °C. The experimental data were then fit to a theoretical model to determine the K_d of the dimer, and this is described in more detail in Results.

Time Dependence of Protease Activity. The wild-type enzyme at 50 nM concentration in the high-salt buffer was incubated for varied lengths of time at 30 °C. Reactions were initiated with the addition of the substrate. The initial velocity for the protease samples was measured by monitoring the hydrolysis of the internally quenched fluorogenic peptide substrate 4-(4′-dimethylaminophenylazo)benzoyl-Arg-Gly-Val-Val-Asn-Ala↓Ser-Ser-Arg-Leu-Ala((2′-aminoethyl)amino-naphthalene-1)sulfonic acid substrate (Bachem) (λ_{ex} = 355 nm and λ_{em} = 495 nm) (*29*).

RESULTS AND DISCUSSION

The Wild-Type Enzyme Maintains Activity in the High-Salt Buffer at 50 nM Concentration. To characterize the time-dependence of the activity of wild-type HCMV protease, we incubated the enzyme at 50 nM concentration for various lengths of time in the high-salt buffer at 30 °C, and then determined the initial velocity of the enzyme for the hydrolysis of the internally quenched fluorogenic substrate in the same buffer (*29*). The results showed that there was only an insignificant decrease in the catalytic activity of the enzyme, even after incubation for 6 h (Figure 2). This demonstrates that wild-type HCMV protease remains dimeric in the high-salt buffer for a significant period of time, which explains why the enzyme can be assayed at this concentration. However, if the K_d of the dimer under this condition is really 0.6−6 μM, as reported earlier, it raises a question as to how the protease can maintain the dimeric association at a concentration significantly below the K_d. It was generally believed that the dimer dissociation rate is very slow (from minutes to hours) (*18*), allowing the activity of protease to be measured even when the concentration is significantly below the K_d. However, our experiment showed little decrease in protease activity even after 6 h of incubation. Earlier studies showed that the dimer may remain stable only for several minutes at 20 °C (*3*, *18*). Our results therefore suggest that the slow dissociation model is unlikely to be correct.

In a previous report, dimers of HCMV protease at 50 nM concentration were observed to dissociate within 40 min in the presence of 20% glycerol at 30 °C (*18*). A comparison with our results would suggest that Na_2SO_4 may play a more effective role in maintaining the protease in the dimer form, and this is confirmed by our dilution experiments (see next).

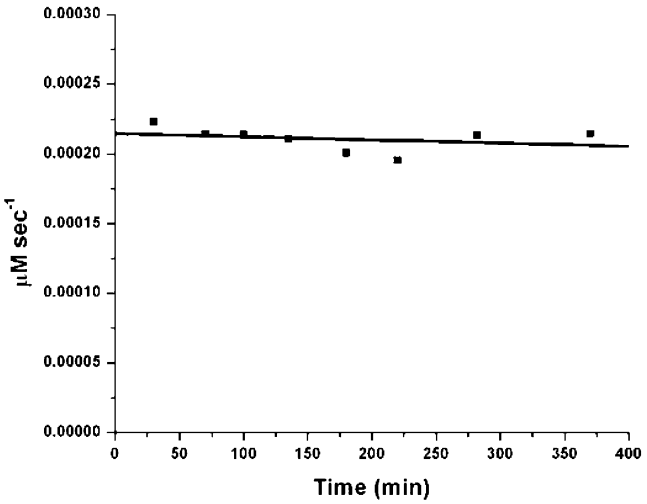

FIGURE 2: Time dependence of HCMV protease activity. The catalytic activity of 50 nM HCMV protease after incubation for various times in the high-salt buffer is shown. There is only a negligible decrease in activity even after 6 h incubation at 30 °C.

In contrast to the results from the high-salt buffer, when wild-type HCMV protease at 50 nM concentration was incubated in the low-salt buffer at 30 °C, the enzyme quickly lost its activity (data not shown). This demonstrates that the dimer of the protease is not stable in the low-salt buffer, and is consistent with the reported high K_d of the dimer (10−60 μM) in this condition. It could also be possible that the conformation of the enzyme is not stable in the low-salt buffer, especially in the monomeric form, and the protease may have become partially unfolded in this condition as well.

0.5 M Na_2SO_4 Enhances the Stabilization of the Dimer. We next performed dilution experiments to study the time course of the dissociation of the HCMV protease dimers. To characterize the effects of various anti-chaotropic agents on this process, we used four different solutions: the low-salt buffer, and the low-salt buffer supplemented with 10% glycerol, or 0.5 M Na_2SO_4, or 0.5 M Na_2SO_4 and 10% glycerol (high-salt buffer). The protease stock solution is diluted into the different solutions, and the progress of the reaction is monitored immediately after the dilution, via the increase in fluorescence due to the cleavage of the fluorogenic substrate (*29*).

In one experiment, a stock solution of HCMV protease at 0.6 μM concentration in the low-salt buffer was diluted into the different solutions to a final concentration of 50 nM (Figure 3A). When the dilution was into the low-salt buffer, little catalytic activity was observed, consistent with the fact that the stock solution contained mostly monomers of the enzyme as its concentration was significantly below the expected K_d. Interestingly, the inclusion of 10% glycerol produced only a small increase in activity (Figure 3A), suggesting that glycerol at 10% concentration may not promote the formation of dimers. In contrast, in the presence of 0.5 M Na_2SO_4, or both 10% glycerol and 0.5 M Na_2SO_4 (the high-salt buffer), the enzyme displayed robust catalytic activity, with little delay after the dilution. Moreover, the reaction appeared to accelerate during the incubation time

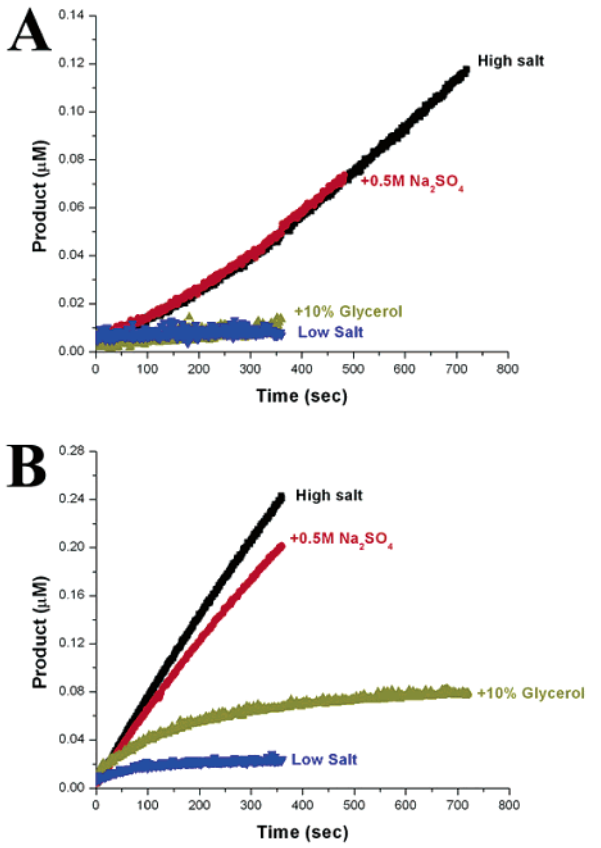

FIGURE 3: Dilution experiments with HCMV protease at 24 °C. (A) HCMV protease in a stock solution at 0.6 μM in a low-salt buffer is diluted into the four different solutions, and the activity of the protease in the hydrolysis of the internally quenched fluorogenic substrate is monitored (*29*). (B) HCMV protease in a stock solution at 60 μM in a low-salt buffer is diluted into the four different solutions, and the reaction progress curves recorded.

(Figure 3A), suggesting that additional dimers might be forming during this time period. Therefore, 0.5 M Na_2SO_4 appears to promote the formation of dimers from monomeric species.

In a different experiment, the stock solution of HCMV protease was at a higher concentration of 60 μM in the low-salt buffer (Figure 3B). Our kinetic measurements suggest that the K_d of the dimer is about 8 μM under this condition (see next), so most of the enzyme was expected to be dimeric in this stock solution. When this solution was diluted into the low-salt buffer, rapid inactivation of the protease was observed, with the enzyme essentially becoming inactive after 1 min (Figure 3B). When the solution contained 10% glycerol, the enzyme was able to stay active for a longer period of time, suggesting that 10% glycerol may be able to stabilize preformed dimers, even though our observations with the 0.6 μM enzyme suggest glycerol does not promote the formation of new dimers (see above). As can be expected, the protease maintained vigorous activity in the presence of 0.5 M Na_2SO_4 (with or without glycerol).

The dilution experiments demonstrate that the dissociation of the dimers of HCMV protease is a facile process in the low-salt buffer, even in the presence of 10% glycerol. On the other hand, in the high-salt buffer, monomers of the protease can quickly associate into dimers (Figure 3A), whereas dimers remain stable (Figure 3B). It should also be noted that the first time point that is observed from these experiments is generally about 10 s after the dilution itself. Any changes occurring during this time period are not recorded in our experiments.

Titration Experiments. To further study the monomer−dimer equilibrium of the protease in solution, we next performed titration experiments, which characterized the monomer exchange between the wild-type protease and the protease carrying the S132A mutation. The S132A mutant is catalytically inactive as the active site nucleophile Ser132 has been disabled.

The basic consideration of the titration experiment is as follows. The wild-type HCMV protease, at a fixed concentration, is incubated overnight with increasing concentrations of the S132A mutant, in the low-salt or the high-salt buffer. The overnight incubation is to ensure that the system can reach equilibrium. In such an incubation mixture, the following equilibria can take place:

$$W + W = W_2$$

$$M + M = M_2$$

$$M + W = MW$$

where W represents the wild-type monomer, and M is the S132A mutant monomer. As the mutation is in the active site, far from the dimer interface (Figure 1A), it is likely that the K_d of the three equilibria above should be essentially the same, since the interface of the MW heterodimer should be essentially the same as that of the W_2 (or M_2) homodimer.

As the dimer interface is unlikely to be disrupted in the MW heterodimer, we expect that this heterodimer should be catalytically active, with a reaction rate one-half of that of the W_2 homodimer. If the concentration of the wild-type protease is significantly below the K_d of the dimer, the enzyme will primarily exist as monomers and should therefore have little catalytic activity by itself. The introduction of an increasing amount of the S132A mutant will drive the wild-type monomers (W) into the MW heterodimer form. This should produce an increase in the activity of the mixture, even though the total concentration of the wild-type protease remains constant.

The K_d of the dimer can be determined from this titration curve. The initial velocity (v) of the reaction is given by

$$v = k([MW] + 2[W_2])$$

where k is a constant that depends on the substrate concentration, and the K_m and k_{cat} of the active site. Given a K_d for the dimer and the total concentrations of the wild-type and S132A mutant enzymes, the concentration of all the species in solution can be calculated by numerical methods. The actual K_d of the dimer is the value that produces the smallest difference between the observed and calculated initial velocities:

$$\sum (v^o - v^c)^2$$

Our experimental observations also rule out the possibility that the MW heterodimer is inactive and that the protease requires two functional active sites in the dimer. Published data suggest that HCMV protease does not have cooperative behavior, as it displays hyperbolic kinetics with respect to enzyme or substrate concentration (*18*, *19*, *23*). As such, it

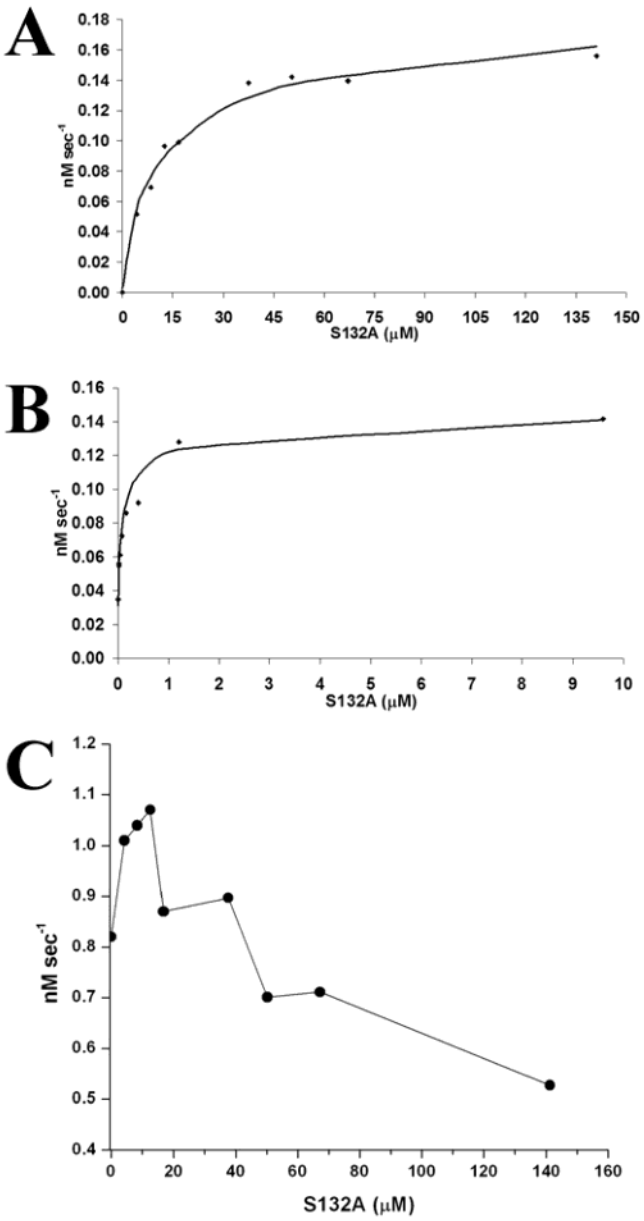

FIGURE 4: Titration experiments with wild-type HCMV protease and the S132A mutant at 24 °C. (A) Wild-type HCMV protease at 50 nM concentration in the low-salt buffer is incubated overnight with increasing concentrations of the S132A mutant. The observed initial velocities (diamonds) are fitted with the model as described in text (curve) to determine the K_d of the dimer. (B) Wild-type HCMV protease at 5 nM concentration in the high-salt buffer is incubated overnight with increasing concentrations of the S132A mutant. The K_d of the dimer can be determined from these data. (C) Wild-type HCMV protease at 50 nM concentration is incubated with higher concentrations of the S132A mutant in the high-salt buffer. A decrease in the activity of the protease is observed, possibly due to the competition of the mutant for binding to the substrate.

is unlikely that there is communication between the two active sites in the dimer.

K_d of the Dimer is 8 μM in the Low-Salt Buffer. Our titration experiment in the low-salt buffer showed that, although the wild-type enzyme had little activity by itself at 50 nM concentration, the introduction of the S132A mutant greatly increased the apparent catalytic activity of the mixture (Figure 4A). The overall activity approaches saturation at high concentrations of the S132A mutant, suggesting that most of the wild-type molecules are engaged in the MW heterocomplex. It also indicates that the S132A mutant has very weak affinity for the substrate in this buffer, as otherwise high concentrations of the S132A mutant should cause a decrease in the initial velocity due to its competition for the substrate (at 2 μM concentration) in the system.

Table 2: Summary of Dimer Dissociation Constants

enzyme	buffer	K_d (μM)
wild-type	high-salt	0.031
wild-type	low-salt	8
S225Y/Y230A homodimer	low-salt	28
S225Y/Y230A:S132A heterodimer	low-salt	5

The K_d for the dimer as determined based on the experimental data is 8 μM (Table 2). The theoretical model has excellent fit to the experimental observations (Figure 4A), confirming that our analysis is generally a good reflection of the actual equilibria in solution. This K_d value is consistent with our observations from the dilution experiments. A protease solution at 0.6 μM concentration contains mostly monomers, whereas a solution at 60 μM contains mostly dimers in the low-salt buffer (Figure 3). This value is also in general agreement with other reported values from kinetic observations, although it is about 10-fold lower than the values observed from analytical ultracentrifugation.

K_d of the Dimer is 31 nM in the High-Salt Buffer. For the titration experiment in the high-salt buffer, the concentration of the wild-type enzyme was lowered to 5 nM. The apparent catalytic activity of the mixture rose quickly with the introduction of the S132A mutant, approaching saturation at 10 μM concentration of the mutant (Figure 4B). The observation of catalytic activity after overnight incubation of the wild-type protease alone at 5 nM concentration (Figure 4B) suggests that the protease is mostly stable in this condition, despite the low concentration. Another evidence for this stability is the fact that full enzyme activity can be restored by the addition of 100 μM S132A mutant after overnight incubation. It might be possible that the S132A mutant prevents the wild-type protease from nonspecifically adsorbing to the sample tube, although this is unlikely. Bovine serum albumin can be used instead of the S132A mutant to distinguish this possibility.

On the basis of these observations, the K_d of the dimer was determined to be 31 nM (Table 2), and the theoretical model produced an excellent fit to the experimental data (Figure 4B). This K_d value clearly explains the observation that the protease can maintain activity at 50 nM concentration in the high-salt buffer (Figure 2). Our K_d value is in good agreement with that of 2 nM in the presence of 25% glycerol, obtained by kinetic measurements on HCMV protease at different concentrations (*19*). It was also observed in that study that the K_d is even lower in the presence of 50% glycerol. Therefore, the K_d of the dimer appears to be very sensitive to the compositions of the solutions.

The activity of the mixture was inhibited by high concentrations (50 μM) of the S132A mutant (Figure 4C). We believe this is because high concentrations of the mutant can compete for binding the peptide substrate in the high-salt buffer, consistent with the lower K_m for the substrate in the high-salt buffer (Table 1, see below). A conformational change in the protease has been observed in the high-salt buffer, and this change may enhance the affinity between the protease and the substrate.

The K_d value in the high-salt buffer as determined from our titration experiments is significantly below those reported from other kinetic studies (*3*), as well as a value of 6 μM from analytical ultracentrifugation (*24, 27*). Several possible reasons can be given to explain this difference. One is that the system has not reached equilibrium in our assay solutions. This is however unlikely, as we have incubated the mixture for 24 h before determining the kinetic activity of the mixture. Another possibility is that the substrate may be able to induce dimerization of the protease, whereas the ultracentrifugation experiments reported earlier were carried out in the absence of substrate.

To address the issue of substrate-induced dimerization, we performed analytical ultracentrifugation (AUC) experiments with the protease in the presence of the internally quenched substrate or the peptidomimetic inhibitor with IC_{50} of less than 50 nM (*17, 31*). On the basis of preliminary AUC experiments, there was no evidence that the presence of the inhibitor could significantly enhance dimer formation. The experiments in the presence of the substrate were more complicated due to the rapid digestion of the substrate by the enzyme, and meaningful data could not be extracted from the observed data. Overall, our studies show that substrate-induced dimerization is likely to have only a minor impact on the monomer−dimer equilibrium of HCMV protease. Additional studies are needed to reconcile the differences in the observed K_d values from the kinetic and the AUC experiments.

Titration Experiment with Dimer Interface Mutant. We next extended our titration experiment to that of the S225Y/Y230A dimer interface mutant. Similar to our observations earlier with the S225Y mutant (*24*), the S225Y/Y230A mutant also has extremely low catalytic activity, but it maintains dimeric association in solution, based on gel-filtration experiments (data not shown). For the titration experiment, we incubated this double mutant with increasing concentrations of the S132A mutant in the low-salt buffer. As the double mutant has very low catalytic activity, it was necessary to use a much higher concentration of 50 μM to obtain a reasonable signal from the kinetic experiments.

To analyze the data from this titration experiment, the three equilibria described above for the S132A/wild-type titration should still apply to this system. However, the K_d values of the three equilibria are expected to be different. In addition, the activity of the S225Y/Y230A monomer in the homodimer is expected to be different from that in the S225Y/Y230A:S132A heterodimer because of differences in their interfaces, such that the initial velocity v is given by

$$v = k(s[\mathrm{MW}] + 2[\mathrm{W}_2])$$

where W represents the S225Y/Y230A monomer, M is the S132A monomer, and s is the activity ratio. In the fitting, the three K_d values and s can be varied systematically to find the best match to the experimental data. To reduce the number of parameters, the K_d for the S132A dimer was fixed at 8 μM, as observed from the wild-type/S132A titration experiments.

Without the addition of the S132A mutant, the S225Y/Y230A mutant is essentially inactive even at 50 μM concentration (Figure 5). However, a greater than 10-fold enhancement of the activity is observed when 240 μM of the S132A mutant is introduced (Figure 5). The K_d values

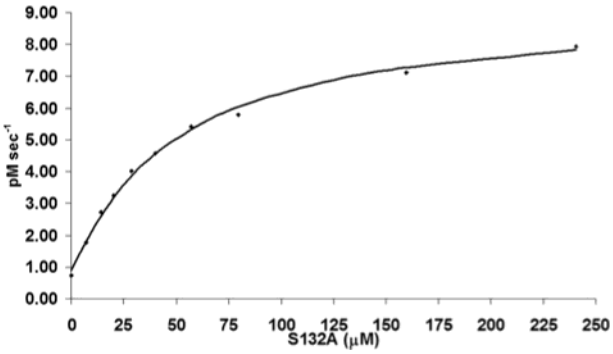

FIGURE 5: Titration experiments with dimer interface mutant and the S132A mutant at 24 °C. The S225Y/Y230A mutant of HCMV protease at 50 μM concentration in the low-salt buffer is incubated overnight with increasing concentrations of the S132A mutant. The observed initial velocities (diamonds) are fitted with the model as described in text (curve) to determine the K_d of the dimers and the relative activity of the S225Y/Y230A monomer in the homo- and heterodimers.

obtained from fitting to the experimental data are 28 and 5 μM for the S225Y/Y230A homodimer and the S225Y/Y230A:S132A heterodimer, respectively (Table 2). The kinetic data suggest that dimers of the S225Y/Y230A double mutant are only slightly less stable than the wild-type dimers, consistent with our earlier studies (*24*). The kinetic data also show that the catalytic activity of the S225Y/Y230A monomer in the heterodimer with S132A is about 7-fold more active ($s = 6.5$) than that in the homodimer, possibly because the interface in the heterodimer has more wild-type characters than the homodimer.

The High-Salt Buffer also Enhances the Catalytic Activity of the Dimer. Using the AMC substrate, we have determined the kinetic parameters of the wild-type enzyme under several different conditions at 24 °C (Table 1). After overnight incubation in the high-salt buffer, the enzyme essentially maintained its overall activity, consistent with observations in Figure 2. The inclusion of 10 μM S132A mutant caused a 3-fold increase in the k_{cat} while having little effect on the K_m (Table 1). This is due to the engagement of all the monomers of the wild-type enzyme in either homodimers or heterodimers, whereas the wild-type enzyme alone at 50 nM concentration will contain appreciable amount of monomeric species ($K_d = 31$ nM). The k_{cat} of 0.39 is likely the true activity of wild-type dimers in the high-salt buffer.

At higher concentration of the S132A mutant, the activity of the mixture is reduced (Figure 4C). This is reflected by a 1.4-fold increase in the apparent K_m, which is remarkably consistent with the reduction in the overall activity of the enzyme from the titration experiment (Figure 4C). This increase in the K_m is due in part to the competition for substrate binding by the S132A mutant in this buffer.

By using the titration experiment, we were able to determine for the first time the kinetic parameters of HCMV protease in the low-salt buffer, in the presence of 67 μM S132A mutant. In this buffer, the protease has a higher K_m and also a lower k_{cat} than in the high-salt buffer, such that the overall activity of the protease is reduced by about 7-fold (Table 1), similar to earlier observations (*19*). There is a large conformational change in the protease in the high-salt buffer (*15, 25, 26*). Our kinetic data suggest that this conformational change both facilitates the binding of the substrate and enhances the efficiency of the catalytic machinery. Structural

studies confirm the induced-fit behavior near the active site of the enzyme (*14*, *16*).

In summary, our dilution and titration experiments show that the dimers of HCMV protease are stabilized in the high-salt buffer. The K_d for the dimer decreases by 260-fold, from 8 μM to 31 nM, going from the low-salt to the high-salt buffer. This is consistent with the structural information showing that most of the dimer interface is hydrophobic in nature (Figure 1B). In addition to stabilizing the dimer, the high-salt buffer also produces a 7-fold enhancement in the catalytic activity of the dimer, as evidenced by the higher k_{cat} and lower K_m in this buffer (Table 1). Earlier studies have suggested a major conformational change in the protease in the high-salt buffer, which may be the structural basis for the activity enhancement.

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