Dimerization of the Human Cytomegalovirus Protease: Kinetic and Biochemical Characterization of the Catalytic Homodimer

Stephen A. Margosiak,*,‡ Darin L. Vanderpool,‡ Wes Sisson,‡ Christopher Pinko,§ and Chen-Chen Kan§

Departments of Biophysics and of Molecular Biology/Biochemistry, Agouron Pharmaceuticals, Inc., 11099 North Torrey Pines Road, La Jolla, California 92037

Received December 1, 1995; Revised Manuscript Received February 22, 1996[⊗]

ABSTRACT: The single-chain 28 kDa human cytomegalovirus (HCMV) protease catalytic domain containing the A143Q mutation has been kinetically and conformationally characterized. The specific activity of the HCMV A143Q protease (HCMVp) increases as the protease concentration increases, suggesting that this protease oligomerizes at high protein concentration to form a more active species. Both cross-linking and light-scattering studies of HCMVp show the existence of a homodimer with an apparent molecular mass of 56 kDa under low ionic strength and high protein concentration. The cosolvent and solute effects of glycerol, trisodium citrate, and NaCl as well as the temperature effects on the HCMVp activity and quaternary structure were investigated. The effects induced by cosolvents and temperature can largely be explained by their influences on the dimerization or oligomerization state of HCMVp. The dissociation constant (K_d) for the HCMVp homodimer was determined to be 8 ± 1 μM with all activity attributed to the dimeric form. Monomeric HCMVp is inactive. This report demonstrates that in vitro, HCMV A143Q protease exists as an obligate catalytic homodimer. This protease dimerization may have regulatory significance during viral replication.

Human cytomegalovirus (HCMV)[1] is a member of the Herpes virus family. The viral genome contains the open reading frame UL80 which encodes the full-length 80 kDa HCMV protease and its substrate, the assembly protein precursor. Full-length HCMV protease cleaves the assembly protein precursor at the M-site as an essential event for capsid assembly during viral replication (Baum et al., 1993). Therefore, HCMV protease is considered to be an attractive target for antiviral chemotherapy. The catalytic domain of the full-length HCMV protease resides between amino acid residues 1 and 256 and is released as a fully active 28 kDa protein via autoproteolysis. However, this catalytic domain can proteolytically cleave itself between amino acid residues Ala-143 and Ala-144 to produce two fragments of 15 and 13 kDa (Burck et al., 1994). The recombinant HCMV protease catalytic domain with a single A143Q mutation was expressed in *Escherichia coli* as a single-chain protein and purified to homogeneity for crystallographic and kinetic studies. This protease was previously shown to be stable and indistinguishable from the wild-type HCMV protease in terms of catalytic activity (Pinko et al., 1995).

The effective design of competitive inhibitors for HCMV A143Q protease (HCMVp) required kinetic characterization of the protein with respect to catalysis and conformational state. For convenient measurement of enzymatic activity, we developed a continuous spectral based assay (Pinko et al., 1995). A resonance energy transfer fluorogenic peptide substrate was designed based on the HCMV protease maturation site cleavage sequence. The long-range acceptor/donor pair, anthranilic acid/3-nitrotyrosine, was employed in this design (Meldal et al., 1991). Cleavage of this peptide results in a large increase in fluorescence at 420 nm when excited at 320 nm. We and others have reported previously that cosolvents and different assay conditions affect the apparent activity and stability of HCMVp (Pinko et al., 1995; Burck et al., 1994). Glycerol, $(Me)_2SO$, DTT, ionic strength, pH, and temperature all have significant impacts on the enzymatic activity. This variation in conditions and the inherent sensitivity of HCMVp activity to them make it difficult to compare data from various laboratories. The disparity in activity may be explained as the occurrence of a conformational transition of the 28 kDa monomeric HCMV protease that is sensitive to assay conditions and that consequently affects the activity. Alternatively, the activity may depend upon the protease's oligomerization state which would also be affected by different assay conditions. This oligomerization may induce conformational changes in the tertiary structure as well.

There are examples of retroviral proteases that occur as catalytic dimers. HIV-1, HIV-2, and RSV proteases dimerize to form a catalytically active species with one active site

* To whom reprint requests and proofs should be addressed. Telephone: 619-622-3000. FAX: 619-622-7998. E-mail: margosiak@agouron.com.

‡ Department of Biophysics.

§ Department of Molecular Biology/Biochemistry.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1996.

[1] Abbreviations: HCMV, human cytomegalovirus; A143Q, alanine-143 to glutamine mutant; kDa, kilodaltons; K_d, dissociation constant; HCMVp, HCMV A143Q protease; HSV-1, herpes simplex virus type-1; DTNB, 5,5′-dithionitrobenzoic acid; HPLC, high-performance liquid chromatography; RET, resonance energy transfer; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; MW_a, apparent molecular weight; $(Me)_2SO$, dimethyl sulfoxide; DTT, dithiothreitol; HIV-1, human immunodeficiency virus type-1; HIV-2, human immunodeficiency virus type-2; RSV, Rous sarcoma virus; *E. coli*, *Escherichia coli*; PK03, RET-fluorogenic HCMVp peptide substrate; $Y(NO_2)$, 3-nitro-L-tyrosine; $k_{cat} = V_{max}/[E]_t$, where V_{max} is the steady-state maximum velocity and $[E]_t$ is the total enzyme concentration; K_m, Michaelis constant; $K_{m,app}$, apparent K_m; k_{obs}, observed rate constant; A_s, specific activity of the dimer; MOPS, 2-(*N*-morpholino)propanesulfonic acid; DSS, disuccinimidyl suberate; Tris, tris(hydroxymethyl)aminomethane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; M, HCMVp monomer; D, HCMVp dimer; E_a, energy of activation; BSA, bovine serum albumin.

0006-2960/96/0435-5300$12.00/0

per dimer (Navia et al., 1989; Wlodawer et al., 1989; Jordan et al., 1992; Holzman et al., 1991; Miller et al., 1989). The monomeric forms of these proteases are inactive in all three cases. This dimerization may play an important role in the regulation of viral replication. Kräusslich recently reported that cells infected with a virus expressing a tethered HIV-1 protease dimer failed to produce mature virions (Kräusslich, 1991). This suggests that for some viruses the dimerization not only is needed for the formation of an active protease but also needs to be temporally coordinated with other biochemical processes during viral replication.

Here, we report the data obtained from biophysical and kinetic studies of HCMVp and demonstrate that HCMVp exists as an obligate catalytic homodimer while the monomeric species is inactive. The affinity between dimeric subunits is determined as is the effect of dimerization on the thermal stability of HCMVp. The dimeric association of the HCMVp catalytic domain leads us to speculate whether dimerization of the full-length UL80 HCMV protease occurs in vivo and serves a purpose in the regulation of protease activity during the viral capsid assembly. If so, the dimerization of HCMV protease and its physiological significance in viral replication may represent a unique regulatory timing mechanism for the Herpes virus lifecycle.

MATERIALS AND METHODS

Production of the HCMV A143Q Catalytic Domain. HCMVp was expressed in *E. coli* as a single-chain protease and purified to high homogeneity as described previously in Pinko et al. (1995). The calculated MW of the protease based on primary sequence is 28 039.

Continuous Resonance Energy Transfer Fluorogenic Peptide Assay. An internally quenched fluorogenic peptide substrate, PK03, was synthesized for us by Enzyme Systems Products, Dublin, CA. PK03 has the sequence anthranilamide-GVVNA-SSRLAY(NO_2)G (Pinko et al., 1995). The anthranilamide group emits fluorescence at 420 nm when excited with 320 nm monochromatic light. The fluorescence of this group is significantly quenched by resonance energy transfer to the 3-nitrotyrosine residue (Meldal et al., 1991). When the peptide is cleaved at the Ala−Ser scissile bond, an increase in the observed fluorescence of the anthranilamide is obtained. The standard 600 μL cleavage reaction was performed at 25 °C in a microfluorescence cuvette containing 25 mM MOPS, pH 7.2, 50% glycerol, 1 mM DTT, 2% $(Me)_2SO$, and 20 μM PK03. The concentration of HCMVp was 30−80 nM so that less than 5% of the substrate was cleaved within the 5 min reaction time. The continuous change in fluorescence was monitored using a Perkin Elmer LS-5B luminescence spectrometer (Norwalk, CT).

For accurate initial rate determination at high HCMVp concentrations (3−24 μM), an Applied Photophysics stopped-flow spectrofluorometer (Leatherhead, U.K.) was employed. Enzyme and substrate were rapidly mixed with pneumatically driven syringes, and illuminated with 320 nm monochromatic light while the emitted light was monitored at wavelengths greater than 330 nm. A UG5 broad band-pass optical filter was used to block second-order scattered light from the monochromator, and a WG330 optical filter in front of the detector blocked Rayleigh scattered light. The average dead time for this mixing is 2.5 ms. Data collection commenced after the occurrence of mixing artifacts. The initial rates were calculated as the increase in fluorescence versus time. Activity can be expressed in units of micromoles of product formed per second using the full fluorescence yield determined after cleavage for each substrate concentration. Determination of the full fluorescence yield is required for each substrate concentration to account for absorptive inner-filter effects on fluorescence. Fluorescence yield determinations were performed for each fluorimeter employed in order to correct for instrumental variation. This allowed direct compilation of specific activity determined with data collected over a wide range of HCMVp concentrations using two spectrofluorometers.

k_{cat}/K_m, k_{cat}, and K_m for Peptide Substrates. The second-order rate constant k_{cat}/K_m was determined by progress curve analysis under standard assay conditions. The concentrations of HCMVp and fluorogenic peptide substrate PK03 were 0.5−1.0 μM and 20 μM, respectively, so that pseudo-first-order kinetics are appropriate. The continuous change in product fluorescence was monitored. Progress curves were allowed to proceed to completion and were fit to an equation describing a first-order single-exponential decay. k_{cat}/K_m was calculated by the expression $k_{cat}/K_m = k_{obs}/[HCMVp]$. The individual values of k_{cat} and K_m were determined by saturation analysis at 700, 30, and 75 nM HCMVp for the 0, 25, and 50% glycerol conditions, respectively. However, measurement of the fluorescence change could not be used for saturation analysis since high concentrations of substrate caused inner filter effects which artifactually decrease the apparent initial rate. Therefore, reverse-phase HPLC was used to quantify the product formed after cleavage of PK03 by HCMVp. For K_m determination at 50% glycerol by the HPLC assay, the standard 100 μL cleavage reaction contained 25 mM MOPS, pH 7.2, 50% glycerol, 1 mM DTT, 2% $(Me)_2SO$, 75 nM HCMVp, and the substrate PK03 ranging in concentration from 25 to 840 μM. The solubility of PK03 under these conditions was linear up to 850 μM. The cleavage reaction was performed at 25 °C and progressed for only 15 min so that the initial rate could be determined for the cleavage reaction while less than 5% of the substrate was cleaved. The reaction was stopped with glacial acetic acid to a final concentration of 2% and then subjected to reverse-phase/perfusion HPLC using a POROS R2H column (Perceptive Biosystems, Cambridge, MA). Products generated by cleavage were resolved from the substrate and other components with a 1−30% acetonitrile gradient in 0.1% TFA/H_20. Initial rates were calculated from product peak areas and fitted to the nonlinear Michaelis−Menten equation using the graphical data analysis program Kaleidagraph from Abelbeck Software, Reading, PA.

Light Scattering. The molecular weight of HCMVp was determined under various buffered conditions. HCMVp at 71 μM (2 mg/mL) was incubated for 30 min. at room temperature (approximately 25 °C) with variable concentrations of MOPS, NaCl, trisodium citrate, $(Me)_2SO$, and glycerol. These solutions were then measured for their ability to scatter light at 25 °C using a DynaPro-801 light scattering/molecular sizing instrument from Protein Solutions, Charlottesville, VA. Apparent molecular weights (MW_a) of the macromolecule as well as percent polydispersion were calculated using either the mono-modal assumption or the bi-modal assumption within the instrumentation software.

Polydispersion values below 15% represent a homogeneous species.

Cross-Linking of the HCMV A143Q Protease. Glutaraldehyde and disuccinimidyl suberate (DSS) (Pierce, Rockford, IL) were used in the cross-linking of HCMVp. Both these reagents are reactive toward primary amines on side chains of amino acids with lysine as the most reactive. The glutaraldehyde reactions included 36 μM (1 mg/mL) HCMVp; 100 mM glutaraldehyde, 35% glycerol, 1 mM DTT, and 25 mM MOPS, pH 7.2, and were incubated on ice for various lengths of time up to 1 h. Reactions were stopped with the addition of Tris, pH 8.5, to a final concentration of 200 mM to inactivate the excess glutaraldehyde by providing reactive amine groups. Similarly, the DSS cross-linking reaction contained 36 μM (1 mg/mL) HCMVp, 720 μM DSS; 25 mM MOPS, pH 7.2, 25% glycerol, 1 mM DTT, and 3% $(Me)_2SO$. DSS was added slowly from a 25 mM stock solution in $(Me)_2SO$. The reaction was incubated for 2 h on ice and then stopped with the addition of Tris, pH 7.5, to a final concentration of 50 mM. Cross-linking reactions were analyzed by reducing SDS−PAGE (Laemmli, 1970) and by enzymatic activity. For activity measurements and isolation of the cross-linked dimer, all reactions were dialyzed extensively against 10 mM MOPS, pH 7.2, 10 mM NaCl, and 1 mM DTT.

RESULTS AND DISCUSSION

Concentration Dependence of HCMV A143Q Protease Specific Activity in the Absence of Glycerol. The dependence of HCMVp specific activity on enzyme concentration was measured over the broad concentration range of 0.03−24 μM under standard conditions and 0% glycerol using a stopped-flow spectrofluorometer for the high concentrations and a standard spectrofluorometer for the low concentrations. All data are the linear initial rates where the amount of substrate cleaved is less than 5%. Initial rate data, when plotted vs HCMVp concentration, display a nonlinear concave-up dependence with a negative intercept (Figure 1). When displayed as a plot of apparent specific activity vs protease concentration (Figure 2A), the data exhibit a hyperbolic-like dependence yielding a 230-fold increase in the apparent specific activity with the increase in HCMVp concentration. The inset to Figure 2A displays the data in log−log scale to decompress the data at low enzyme concentrations. For monomeric enzymes, or for those that are dimeric where the dissociation constant is in the picomolar range, the specific activity would be constant over all practical concentration ranges provided that initial rates were determined and the protein was stable to denaturation and did not adsorb to cuvette walls. These criteria were satisfied for this protease since initial rates were collected and addition of BSA to reactions did not affect initial rates. Therefore, we speculated that this hyperbolic-like increase in apparent specific activity might be due to the formation of a more active HCMVp dimer. The value for the dimerization K_d can be determined by analysis of these data with the assumption that since [PK03] $\ll K_m$, the apparent K_m ($K_{m,app}$) for PK03 does not change due to the shift in monomer−dimer equilibrium, and that this equilibrium is not perturbed by substrate binding. Consequently, the monomer to dimer equilibrium can be represented by eqs 1 and 2 where M is monomer, D is dimer, and K_d is the equilibrium dissociation constant. Equation 3 expresses monomer concentration in terms of K_d and $[E]_t$. Alternatively, eq 4 expresses dimer concentration in terms of K_d and $[E]_t$. Furthermore, we can express the total enzyme concentration as $[E]_t = [M] + 2[D]$ and the dimer concentration as $[D] = ([E]_t - [M])/2$.

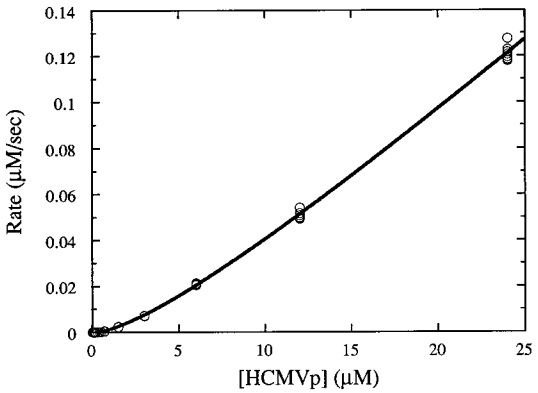

FIGURE 1: Nonlinear dependence of HCMV A143Q protease activity on enzyme concentration. HCMVp activity was measured using the RET-fluorogenic substrate PK03. The reactions contained 25 mM MOPS, pH 7.2, 1 mM DTT, 2% $(Me)_2SO$, 30 μM PK03, and 0.03−24 μM HCMVp. Initial rates were collected at 25 °C. However, initial rates for enzyme concentrations less than 0.06 μM were undetectable at this substrate concentration. The data presented are a compilation of rate data collected on two fluorometers. Rates using high concentrations of enzyme were collected with a stopped-flow spectrofluorometer over very short time frames. Rates using low concentrations of enzyme were collected with a standard fluorometer. The rates were normalized as described under Materials and Methods. Normalized rates were fitted to eq 5 as described under Results to yield a dimerization K_d of 8 ± 1 μM and a maximal specific activity of 0.0076 μM μM^{-1} s^{-1}. The curve represents the best fit of eq 5 to the data.

$$M + M \leftrightarrow D \quad (1)$$

$$K_d = M^2/D \quad (2)$$

$$[M] = 1/2[-K_d/2 + (K_d^2/4 + 2K_d[E]_t)^{0.5}] \quad (3)$$

$$[D] = 1/8[K_d + 4[E]_t - (K_d^2 + 8K_d[E]_t)^{0.5}] \quad (4)$$

$$\nu = A_s[D] = A_s(1/8[K_d + 4[E]_t - (K_d^2 + 8K_d[E]_t)^{0.5}]) \quad (5)$$

If we assume that all activity is due to the dimer then the observed activity (ν) is defined by eq 5 where A_s is the specific activity of the dimer. The data in Figure 1 were fitted to eq 5 and yielded a maximal specific activity of 0.0076 ± 0.0002 μM μM^{-1} s^{-1} and a dimerization K_d of 8 ± 1 μM.

The monomer form of HCMVp is essentially inactive. In Figure 2A, notice that at no point, as we approach very low protease concentrations, is the specific activity of the protease independent of its concentration. Based on these results presented above, we can calculate the relative concentrations of monomer and dimer by eqs 3 and 4 and the activity attributed to each. This analysis indicates that the HCMVp monomer must be inactive or below the limits of detection since there is no activity unaccounted for over that calculated from the concentration and specific activity of the dimer by eq 5. Furthermore, we attempted to fit the data in Figure 1

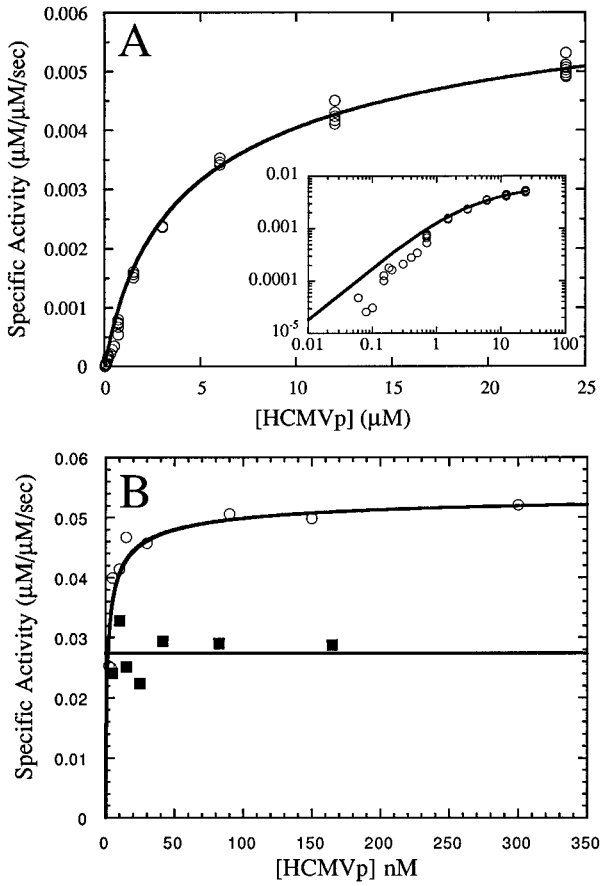

FIGURE 2: Dependence of HCMV A143Q protease specific activity on enzyme concentration. (A) Specific activity in the absence of glycerol. The inset is a log−log plot of the same data in order to decompress the X and Y axes. The apparent specific activity curve without glycerol was calculated using eqs 4 and 5 with the fitted parameters, K_d, and the maximal specific activity from Figure 1. This simulated curve (—) is overlaid on the experimental data (○) to clearly show the excellent agreement. The slight variation of the data from the simulated curve in the inset is presumably a result of a small error in the scaling between the two fluorometers and is exaggerated by the log−log scale. (B) Specific activity in 25% (○) and 50% glycerol (■). Rates were collected with a standard fluorometer over an enzyme concentration range of 5−300 nM and 2.5−165 nM for 25 and 50% glycerol, respectively. The 25% glycerol data were fitted to eq 5 to yield a dimerization K_d of 1.9 ± 0.7 nM and a maximal specific activity of 0.055 ± 0.001 μM μM^{-1} s^{-1}. The curve shown is simulated as before, while the line drawn through the 50% glycerol data represents the average specific activity of 0.027 μM μM^{-1} s^{-1}.

to the expression which included a term for a finite activity of the monomer. However, the fitted activity was essentially zero with a high degree of error. Simulations of specific activity assuming a monomer/dimer equilibrium with a K_d of 8.4 μM were accomplished and showed excellent agreement with the hyperbolic-like shape of the experimental apparent specific activity data (Figure 2A), demonstrating the nonlinear increase in specific activity with enzyme concentration. The large increase in the apparent specific activity with the increase in HCMVp concentration together with the absence of activity of the HCMVp monomer demonstrates that HCMVp must dimerize for it to be catalytically competent.

Effect of Glycerol on HCMV A143Q Protease Catalytic Activity and Dimerization. Additional evidence supporting catalytic dimer formation was the observed effect of glycerol on the activity of HCMVp. In our previous report (Pinko et al., 1995), we showed a glycerol-dependent 20-fold increase of the amount of product formed by a fixed concentration of protease during a 15 min HPLC assay. However, these data did not reflect the true magnitude of the increase since substrate conversion exceeded 5% at higher glycerol concentrations. Therefore, when repeated at low HCMVp concentrations with less than 5% turnover, the protease activity increased more than 100-fold with the addition of glycerol up to 30%. We speculated that the glycerol-induced increase in activity was a result of dimer stabilization. Consequently, the dependence of the apparent specific activity of HCMVp at nanomolar concentrations was determined in the presence of 25% glycerol. The data again suggested a concentration dependence of the apparent specific activity over this low concentration range (Figure 2B). The dimerization K_d approximated from fitting these data as before is 1.9 ± 0.7 nM. More precise determination of the K_d is difficult since the rates measured at these low protease concentrations were quite low and prone to error. It is clear that 25% glycerol stabilizes the dimer form of HCMVp and lowers the K_d for the monomer to dimer equilibrium from 8 μM to 1.9 nM. Indeed, the specific activities measured in the presence of 50% glycerol remained constant for all concentrations of HCMVp tested, suggesting that the higher glycerol concentration caused a further decrease in the dimer K_d (Figure 2B). The maximal specific activity increases in 25% and 50% glycerol by 9-fold and 4.5-fold, respectively, suggesting an apparent glycerol effect on the k_{cat} and K_m as well as on dimerization.

Table 1: Effect of Glycerol Concentration on k_{cat}, K_m, and k_{cat}/K_m for HCMV A143Q Protease and PK03[a]

[glycerol] (%)	k_{cat} (min^{-1})	K_m (μM)[b]	k_{cat}/K_m (μM^{-1} min^{-1})[c]
0	nd[d]	>850	0.048 ± 0.006[d] (0.0044 ± 0.0005)[d]
25	35 ± 2[e]	125 ± 33	0.28 ± 0.03
50	22 ± 3[e]	134 ± 15	0.165 ± 0.009

[a] Assays were performed in 25 mM MOPS, pH 7.2, 1 mM DTT, 2% $(Me)_2SO$, and variable glycerol concentrations at 25 °C. [b] For K_m determination, 700 nM, 30 nM, and 75 nM HCMVp were used, respectively, in assay conditions containing 0%, 25%, and 50% glycerol. [c] For k_{cat}/K_m determination, the HCMVp concentrations were 0.5−3.6 μM. [d] The k_{cat} and K_m for 0% glycerol conditions could not be determined since the apparent K_m exceeded the solubility limit of the substrate (850 μM). k_{cat}/K_m was calculated based on the calculated [dimer] from K_d and $[E]_t$. The value in parentheses is k_{cat}/K_m calculated from $[E]_t$. [e] The k_{cat} value for 25% and 50% glycerol conditions was derived from the k_{cat}/K_m and the K_m values determined by progress curve analysis and saturation analysis, respectively.

The glycerol effects on the activity of HCMVp were investigated further. k_{cat}/K_m, k_{cat}, and K_m in 50% and 25% glycerol were determined for the PK03 peptide substrate while for 0% glycerol only the k_{cat}/K_m was determined by both progress curve analysis and the dependence of initial rates on very low substrate concentrations (Table 1). However, all enzyme present is not in the dimer form at 0% glycerol as it is in 25 and 50% glycerol; thus, the value of k_{cat}/K_m for 0% glycerol was calculated using the active dimer concentration obtained from $[E]_t$ and K_d. Individual values of k_{cat} and K_m were not obtained since the apparent K_m was higher than the solubility limit of PK03. This high K_m is an apparent value due to the effects of substrate binding on the dimerization equilibrium at low concentrations of enzyme. If one considers the applicable reaction pathway (Scheme 1), one would expect that increasing concentrations of

substrate (S) would shift the dimer equilibrium to the right due to the formation of the DS species. This shift would increase the measured K_m and V_{max} to higher apparent values since the concentration of catalytically competent dimer is increased as the concentration of S increases.

Scheme 1

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DS \xrightarrow{k_3} D + P$$

However, concentrations of glycerol above 30% lead to a decrease in activity. This loss of activity is reflected in the lower k_{cat} at 50% glycerol than at 25% glycerol (Table 1). This suggests that glycerol may decrease the true k_{cat} due to its viscosity effect and that the k_{cat} at 0% glycerol may be greater than 35 min^{-1} based on dimer concentration. However, it is impractical to determine k_{cat} and K_m at concentrations of enzyme high enough so that all enzyme present is in the dimer form. There are other factors that could contribute to the apparent decrease in K_m in the presence of glycerol. Glycerol may stabilize the Michaelis complex, thus truly lowering the K_m. Furthermore, the fact that glycerol stabilizes the dimer indicates that it affects the magnitudes of the microscopic rate constants for the dimerization (k_1) and monomerization (k_{-1}) of the protease, and thus since K_m represents a collection of rate constants including k_1 and k_{-1}, the effect on K_m is an apparent one.

The ability of glycerol to enhance the catalytic activity of HCMVp is a commonly observed phenomenon which was also observed by others with the wild-type HCMV and HSV-1 proteases (Burck et al., 1994; O'Boyle et al., 1995; Hall & Darke, 1995). This commonality suggests that different proteases from the Herpes virus family may share similar structural and biochemical properties and that the HSV-1 protease may dimerize as well.

Effect of Salts on HCMV A143Q Protease Catalytic Activity. NaCl and trisodium citrate both enhance the activity in the absence of glycerol (Figure 3). The NaCl data are expanded in the inset to Figure 3. However, citrate enhances the activity 290-fold and much more efficiently than does NaCl. It was of interest to determine if ionic strength was the major contributor to this effect. The ionic strengths of NaCl and trisodium citrate solutions were calculated from the equation $I = {}^1/_2\sum c_i(Z_i)^2$ to show that ionic strength is a minor factor affecting the HCMVp activity in the absence of glycerol. The activity in the presence of equal ionic strengths of NaCl and trisodium citrate is much higher for trisodium citrate. The k_{cat}/K_m was determined, with PK03 and in the presence of 800 mM trisodium citrate, to be 1.24 μM^{-1} min^{-1}. This value is 7.5-fold higher than the k_{cat}/K_m in 50% glycerol.

Trisodium citrate appears to positively affect another member of the Herpes virus protease family. Recently, Hall and Darke reported the activation of the HSV-1 protease by anions such as phosphate and citrate or those that have been termed kosmotropes. Kosmotropes, or water structure-forming solutes, are known to cause salting-out and aggregation effects with proteins. These authors speculated that the lower K_m values measured for peptide substrates in the presence of 0.2 and 0.8 M citrate could be explained by anion destabilization of free or unbound peptide substrate in solution and subsequent stabilization of the Michaelis complex. Unfortunately, to date very little is known about the involvement of water molecules in mediating substrate binding to Herpes proteases. However, these authors also suggested that based on citrate anion-dependent changes in intrinsic protein fluorescence and proteolytic sensitivity, a change in the conformational state occurs which affects substrate binding. Although the effect of trisodium citrate on the dimerization K_d of HCMVp was not determined, our light scattering results (Table 2) with this protease revealed the formation of aggregated HCMVp at 1.0 M trisodium citrate, correlating with an increase in activity. Therefore, it is quite likely that a dimerization event may also occur for HSV-1 protease as well and the apparent K_m effects may as well be due to the dimerization equlibrium as in HCMVp. This quaternary protein structural change that affects the catalysis of amide bond hydrolysis may be a general property of the Herpes virus proteases.

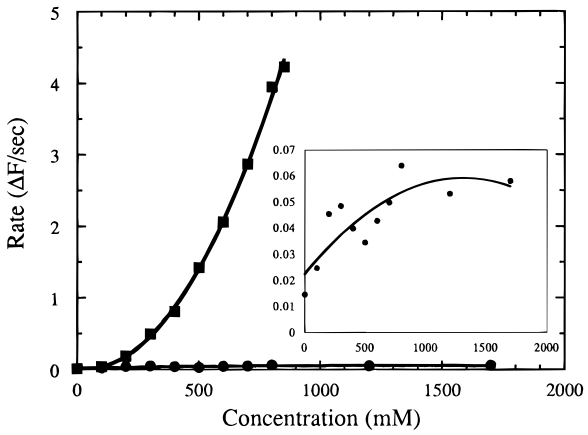

FIGURE 3: Effect of trisodium citrate and NaCl on HCMV A143Q protease activity. HCMVp activity was measured using the RET-fluorogenic substrate PK03. The reactions contained 25 mM MOPS, pH 7.2, 1 mM DTT, 2% $(Me)_2SO$, and 200 nM HCMVp. Trisodium citrate (■) and NaCl (●) were included at 0−850 mM or 0−1700 mM, respectively. Trisodium citrate solutions were adjusted to pH 7.2 with NaOH. Glycerol was absent from these assays. The reaction was started with the addition of enzyme, and initial rates were determined at 25 °C. Increases in ionic strength did not significantly affect the fluorescence yield. The smooth curves fitted to the data were generated by a second-order polynomial equation. The inset displays NaCl (●) data with an expanded *y*-axis.

Table 2: Effect of Cosolvents and Solutes on the Apparent Molecular Weight of HCMV A143Q Protease Determined by Light Scattering[a]

cosolvent or solute	MW_a	oligomer[b]	polydispersion (%)
low ionic strength, pH 7.2	56 000	dimer	10
1 M NaCl	67 000	dimer	21
20% $(Me)_2SO$	120 000−140 000	tetramer	39
20% glycerol	62 000	dimer	NA[c]
1 M trisodium citrate	520 000	aggregate	56

[a] Light scattering experiments were performed with 70 μM HCMVp at 25 °C, pH 7.2, and the data were fitted using a monodisperse assumption. [b] Apparent oligomerization states based on MW_a. [c] NA, not applicable. Polydispersion cannot be calculated in the presence of glycerol.

Molecular Weight of HCMV A143Q Protease by Light Scattering. Light scattering was used to determine the apparent molecular weight (MW_a) of HCMVp. The MW_a of the protease at 71 μM in a low ionic strength buffer is

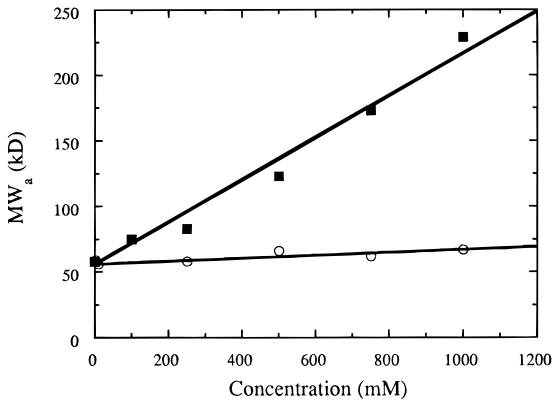

FIGURE 4: Effect of trisodium citrate and NaCl on HCMVp apparent molecular weight. HCMVp was incubated at 70 μM in 25 mM MOPS, pH 7.2, 1 mM DTT, and variable concentrations of trisodium citrate (■) and NaCl (○) for 30 min at room temperature prior to molecular weight determination by light scattering. Straight lines are fitted to the data.

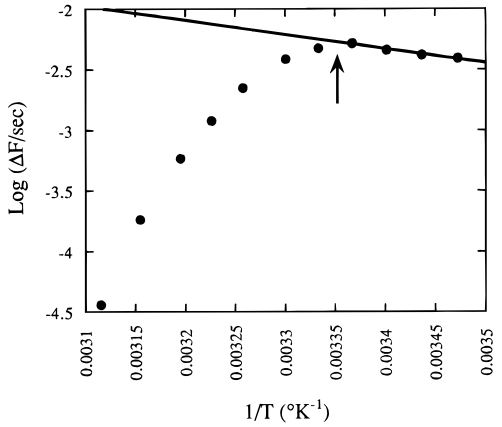

FIGURE 5: Effect of temperature on the activity of HCMV A143Q protease. HCMVp activity was measured using the RET-fluorogenic substrate PK03. The reactions contained 25 mM MOPS, pH 7.2, 1 mM DTT, 2% $(Me)_2SO$, and 1 μM HCMVp. Activity was measured in a stopped-flow spectrophotometer at the temperature indicated. The rate data are expressed as an Arrhenius plot. The apparent activation energy (E_a) was determined by a linear fit through data collected at less than 25 °C. The arrow indicates 25.5 °C, the temperature at which the slope breaks.

56 000 with 10% polydispersion. This low (10%) polydispersion indicates that the dimeric HCMVp exists homogeneously as one single species. We questioned whether a disulfide linkage was responsible for the dimerization of HCMVp since the native protease has five cysteine residues, three of which are exposed (Burck et al., 1994; Pinko et al., 1995). Our results suggest that the HCMVp dimer is not linked by disulfide bonds since scattering experiments completed in the presence of 10 mM DTT did not show the dissociation of the dimer. In addition, further experiments that demonstrated the presence of oligomers contained 1 mM DTT.

The effects of cosolvents and solutes on the HCMVp MW_a are presented in Table 2. Addition of glycerol up to 20% increases the MW_a slightly to 62 000. In this case, the polydispersion cannot be determined due to the scattering characteristics of glycerol. This MW_a largly represents a dimeric species. Addition of $(Me)_2SO$ up to 20% leads to aggregation of HCMVp with the average MW_a of a tetramer with high polydispersion. Previously, $(Me)_2SO$ was shown to enhance the activity without glycerol present but inhibits activity, as does NaCl, in its presence (Pinko et al., 1995). As shown in Figure 4 and Table 2, the addition of NaCl to HCMVp leads to quite small increases in both MW_a and polydispersion. However, the addition of trisodium citrate to the final concentration of 1.0 M leads to large increases in MW_a up to 520 kDa representing aggregates (Figure 4, Table 2). Also, the polydispersion increased dramatically, indicating the heterogeneity of the oligomerized protease species. The high polydispersion seen may be an effect of the mono-modal fitting routine used. Fitting the data using a two-component or bi-modal model did not improve the fit statistics and could not isolate two major species. It is clear that nonspecific aggregation occurs at high trisodium citrate concentrations. Interestingly, the activity of HCMVp increases significantly in this concentration range of trisodium citrate and correlates quite nicely with the MW_a. This may indicate that this protein, while stable as a dimer, prefers to be present as a higher aggregate.

Temperature, Activity, and the Arrhenius Relationship. The temperature dependence of HCMV A143Q protease catalytic activity was investigated. The catalytic constant k_{cat}/K_m was determined at 20, 25, and 37 °C to be 0.152, 0.162, and 0.0645 μM^{-1} min^{-1}, respectively, for HCMVp with the peptide substrate PK03. The small difference in k_{cat}/K_m between 20 and 25 °C and the marked loss in activity at 37 °C prompted us to look at the Arrhenius relationship in more detail. Initial rate data collected at various temperatures are presented as an Arrhenius plot (Figure 5). The data shows striking nonlinearity with a maximum of the curve at ~25 °C. Protein unfolding normally does not occur at this low temperature since thermal denaturation usually transpires at 50−60 °C. We have determined that this temperature-induced loss in activity is rapid and is fully reversible by cooling an enzyme sample which was heated to 40 °C back to 25 °C. This reversibility reveals that the increased temperature may simply dissociate the dimer without thermal denaturation. As expected, when the enzyme sample was heated to 50 °C, the reversibility is incomplete due to the true thermal denaturation of the monomer.

The activation energy (E_a) calculated from the slope of the Arrhenius plot below 25 °C is 5340 cal/mol. This E_a is below the generally expected value of 10 000 cal/mol for most proteins within this temperature range (Segel, 1975). The nonlinearity, reversibility, and low E_a indicate that this relatively small increase in temperature may have a profound and detrimental effect on the quaternary structure of HCMVp. The sharp transition is presumably due to the dissociation of the active dimer as temperature is increased rather than thermal denaturation. Another plausible explanation would be a temperature-induced increase in the K_m for the substrate. However, the expected changes in K_m are small compared to the large loss in activity. In fact, we measured the $K_{m,app}$ at 25 and 35 °C in 50% glycerol and found it increases by a small factor of 1.6-fold. The apparent K_m in the absence of glycerol is immeasurably high but would be expected to show the same increasing trend. The temperature-induced increase in k_{cat} should more than offset the K_m effect which would simply decrease the slope of the Arrhenius plot. The low apparent value of E_a calculated between 15 and 25 °C may be in part due to this K_m shift. The additional sharp loss in activity with increasing temperature is well below

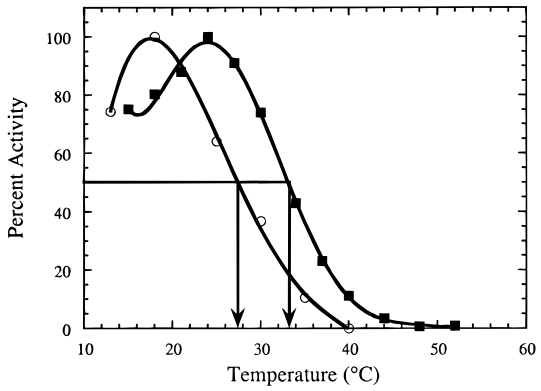

FIGURE 6: Effect of HCMV A143Q protease concentration on the $T_{1/2}$ of thermal inactivation. HCMVp activity was measured using the RET-fluorogenic substrate PK03. The reactions contained 25 mM MOPS, pH 7.2, 1 mM DTT, 2% $(Me)_2SO$, and either 1(○) or 26.5 μM (■) HCMVp. Activity was measured in a stopped-flow spectrophotometer at the temperature indicated. The rate data from the two concentrations were normalized to percent maximal activity since the specific activity at 26.5 μM protease is ~4× that at 1 μM protease. The curves fitted to the data were generated with a fourth-order polynomial equation. The arrows indicate the values of $T_{1/2}$ of 27 and 33 °C for 1 and 26.5 μM HCMVp, respectively.

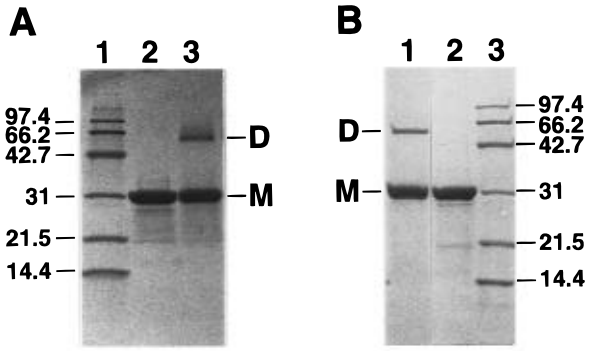

FIGURE 7: Cross-linking of the HCMV A143Q protease homodimer. Cross-linking reactions were performed under conditions to promote dimer formation as described under Materials and Methods. (A) Reducing SDS−PAGE of the glutaraldehyde cross-linked HCMVp; 5.6 μg of protein was loaded into each lane. Lane 1, Low molecular weight standards; lane 2, HCMVp prior to cross-linking; lane 3, HCMVp cross-linked in the presence of glycerol. (B) Reducing SDS−PAGE of the disuccinimidyl suberate (DSS) cross-linked HCMVp; 5.0 μg of protein was loaded into each lane. Lane 1, HCMVp cross-linked in the presence of glycerol; lane 2, HCMVp prior to cross-linking; lane 3, low molecular weight standards.

the temperature normally associated with thermal denaturation of monomeric proteins. Oligomeric proteins are generally less stable to thermal denaturation than monomeric proteins but quite stable at room temperature. Therefore, in the absence of glycerol, the HCMVp dimer is significantly dissociated at >25 °C.

Effect of HCMV A143Q Protease Concentration on the $T_{1/2}$ of Dissociation. $T_{1/2}$ is the temperature at which 50% of the macromolecules have become inactive. Normally the $T_{1/2}$ for thermal denaturation is constant and independent of the concentration of monomeric proteins as well as those which undergo a thermal-induced conformational change. If a dimer to monomer equilibrium is present, then $T_{1/2}$ will be dependent upon protein concentration since increasing protein concentration from below to above the dimerization K_d perturbs the equilibrium toward the more stable dimer. As dimer is formed, the overall stability would increase and thus raise the $T_{1/2}$ of inactivation. As shown in the thermal inactivation plot of HCMVp (Figure 6), the $T_{1/2}$ is 27 °C at 1 μM and increases to 33 °C at 26.5 μM HCMVp. $T_{1/2}$ indeed increased at the higher concentration of HCMVp, indicating that a more stable dimeric species is formed. Furthermore, glycerol increases the thermal stability of HCMVp to a $T_{1/2}$ of 42 °C by stabilizing the dimeric protease against heat dissociation and concomitant loss in activity (data not shown).

Cross-Linking of HCMV A143Q Protease Specifically into Dimers. The glutaraldehyde cross-linking reaction was conducted under conditions favorable to dimerization such as the presence of glycerol, low ionic strength, and a high protein concentration at pH 7.2. Glutaraldehyde and DSS were used to cross-link the dimer form under conditions where the dimer is prevalent. Glutaraldehyde functions as a cross-linker with variable spacer lengths since it can react as a monomer or as oligomers. This feature should maximize the amount of cross-linking observed. Although a small amount of non-specifically aggregated protein may be present under these conditions as indicated by light-scattering experiments, this minimal nonspecific aggregation was not expected to result in cross-linked multimers. After glutaraldehyde-mediated cross-linking was accomplished, the reaction was analyzed by SDS−PAGE and showed that only dimer was formed. However, the amount of dimer was small compared to the amount of residual monomer (Figure 7A). Changes in the intrinsic protein fluorescence during a cross-linking reaction showed that the reaction is rapid and finishes in less than 2 min at 25 °C. Therefore, we believe that incubation on ice for 1 h is enough time for cross-linking to finish. The incomplete cross-linking may be a consequence of nonspecific derivatization causing a rapid dissociation of the dimer significantly faster than cross-linking. Nevertheless, the absence of cross-linked multimers suggests that cross-linked dimer formation is due to specific dimer interactions and not to nonspecific aggregation. This result is consistent with the data obtained from light scattering experiments. The homobifunctional cross-linking reagent disuccinimidyl suberate was used to cross-link the dimer with somewhat greater success (Figure 7B). This cross-linker has a spacer length of 11.4 Å. The cross-linking is specific in that again only dimer was formed. There was no evidence for cross-linking into multimers greater than dimer. After extensive dialysis, HCMVp in the glutaraldehyde and DSS cross-linking reactions was inactive. It is not uncommon that modified enzymes lose activity since cross-linkers react with all available reactive side chains. Glutaraldehyde could potentially react with all exposed amine groups on the protein. Furthermore, we have confirmed findings made by others that reaction of the protein with cysteine modification reagents like IAA, DTNB, and NEM will inactivate the protease quite easily (Burck et al., 1994; Margosiak, unpublished results). These reagents react with one or more of the three exposed cysteine residues and presumably disrupt important interactions involved in either protein oligomerization, folding, or substrate binding. Modification of exposed amines appears to result in a similar effect on the activity.

Whether or not the HCMVp dimer contains one or two active sites is currently being investigated. It is of interest to determine if the HCMV protease will form one active site from two subunits as seen with the HIV-1 protease. An

aspartic acid from each subunit is required for a functional HIV-1 protease (Wlodawer et al., 1989; Navia et al., 1989; Cheng et al., 1990). A single competent active site per dimer would generate the possibility of designing inhibitors that target the complete active site and a separate class of inhibitors that target the inactive monomer in order to inhibit dimerization. Dissociative inhibitors have been pursued for HIV-1 protease (Zhang et al., 1991; Babé et al., 1992).

The K_d for the dimerization of the HCMVp catalytic domain seems unusually high. However, this weak dimerization could be an artifact of working with a catalytic domain. Interaction of the full-length HCMVp with itself, in situ, was recently demonstrated by our colleagues using a yeast two-hybrid system (Zalman, personal communication) developed by Chien et al. (1991) to detect protein interactions. This result suggests that the self-association of the full-length protease is appreciably tighter and may have been enhanced by the presence of the extra sequence outside the catalytic domain. We speculate that the K_d for the self-interaction of the full-length HCMVp would be much lower than that determined for the HCMVp catalytic domain and may be less affected by cosolvents and temperature. This strengthening of the self-interaction may bring the K_d into a more reasonable range for in vivo dimerization, and therefore the full-length HCMV protease dimer would be fully active at the physiological temperature of 37 °C.

The in vivo dimerization of HCMV protease may have an important physiological role in the temporal regulation of biochemical processes during viral replication. It is known that during capsid maturation the assembly protein precursor is the major component of a multimeric scaffold structure (Gibson & Roizman, 1972, 1974; Perdue et al., 1974). One can describe the capsid scaffolding as a large ordered protein aggregate. After capsid formation, proteolysis of the assembly protein precursor leads to the dissolution of the scaffold structure and subsequent entry of the viral genome. This critical proteolytic event may be controlled by the oligomerization or aggregation state of the full-length protease and its substrate. It is conceivable that the monomeric protease produced in the cytoplasm remains inactive and that the HCMV protease becomes activated and performs its proteolytic function only when the scaffold is assembled and covered with coat protein. This may represent a unique regulatory mechanism for viral replication.

ACKNOWLEDGMENT

We thank Dr. L. Zalman for sharing unpublished results, Dr. K. Appelt and Dr. J. E. Villafranca for helpful discussion, and Tom Huffaker for technical assistance.

REFERENCES

Babé, L. M., Rosé, J., & Craik, C. S. (1992) *Protein Sci. 1*, 1244.
Baum, E. Z., Bebernitz, G. A., Hulmes, J. D., Muzithras, V. P., Jones, T. R., & Gluzman, Y. (1993) *J. Virol. 67*, 497.
Burck, P. J., Berg, D. H., Luk, T. P., Sassmannshausen, L. M., Wakulchik, M., Smith, D. P., Hsiung, H. M., Becker, G. W., Gibson, W., & Villarreal, E. C. (1994) *J. Virol. 68*, 2937.
Cheng, Y. E., Yin, F. H., Foundling, S., Blomstrom, D., & Kettner, C. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9660.
Chien, C. T., Bartel, P. L., Sternglanz, R., & Fields, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9578.
Gibson, W., & Roizman, B. (1972) *J. Virol. 10*, 1044.
Gibson, W., & Roizman, B. (1974) *J. Virol. 13*, 155.
Hall, D. L., & Darke, P. L. (1995) *J. Biol. Chem. 270*, 22697.
Holzman, T. F., Kohlbrenner, W. E., Weig, D., Rittenhouse, J., Kempf, D., & Erickson, J. (1991) *J. Biol. Chem. 266*, 19217.
Jordan, S. P., Zugay, J., Darke, P. L., & Kuo, L. C. (1992) *J. Biol. Chem. 267*, 20028.
Kräusslich, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 3213.
Laemmli, U. K. (1970) *Nature 227*, 680.
Meldal, M., & Breddam, K. (1991) *Anal. Biochem 195*, 141.
Miller, M., Jaskolski, M., Rao, J. K. M., Leis, J., & Wlodawer, A. (1989) *Nature 337*, 576.
Navia, M. A., Fitzgerald, P. M. D., Mckeever, B. M., Leu, C. T., Heimbach, J. C., Herber, W. K., Sigal, I. S., Darke, P. L., & Springer, J. P. (1989) *Nature 337*, 615.
O'Boyle, D. R., II, Wager-Smith, K., Stevens, J. T., III, & Weinheimer, S. P. (1995) *J. Biol. Chem. 270*, 4753.
Pinko, C., Margosiak, S. A., Vanderpool, D., Gutowski, J. C., Condon, B., & Kan, C. C. (1995) *J. Biol. Chem. 270*, 22634.
Perdue, M. L., Kemp, M. C., Randall, C. C., & O'Callaghan, D. J. (1974) *Virology 59*, 201.
Segel, I. H. (1975) *Enzyme Kinetics*, p 941, John Wiley & Sons, New York.
Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science 245*, 616.
Zhang, Z. Y., Poorman, R. A., Maggiora, L. L., Heinrikson, R. L., & Kézdy, F. J. (1991) *J. Biol. Chem. 266*, 15591.

BI952842U