Dimerization and Activation of the Herpes Simplex Virus Type 1 Protease*

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The quaternary state of the herpes simplex virus type 1 (HSV-1) protease has been analyzed in relation to its catalytic activity. The dependence of specific activity upon enzyme concentration indicated that association of the 27-kDa subunits strongly increased activity. Size-exclusion chromatography identified the association as a monomer-dimer equilibrium. Isolation of monomeric and dimeric species from a size-exclusion column followed by immediate assay identified the dimer as the active form of the enzyme.

Activation of the protease by antichaotropic cosolvents correlated with changes in the monomer-dimer equilibrium. Thus, dimerization of the enzyme was enhanced in solvents containing glycerol or the anions citrate or phosphate. These are substances previously identified as activators of HSV-1 protease (Hall, D. L., and Darke, P. L. (1995) J. Biol. Chem. 270, 22697–22700). The relative potencies of these cosolvents as enzyme activators correlated with their efficiency in promoting dimerization. Under all solvent conditions examined, the dependence of specific activity upon enzyme concentration was consistent with a kinetic model in which only the dimer is active. Dissociation constants for the HSV-1 protease dimer determined with this model at 15 °C, pH 7.5, were 964 and 225 nm in 20% glycerol with 0.2 and 0.5 mM citrate present, respectively. The activation of the HSV-1 protease by antichaotropic cosolvents was hereby shown to be similar in nature to the activation of the other well characterized herpesvirus protease, that from human cytomegalovirus.

Viruses of the herpes family encode a protease essential for viral capsid formation and viral replication (1, 2). The best characterized proteases of this group are those from herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (hCMV) (3–8). During viral assembly, these enzymes are synthesized as precursor proteins that undergo autoproteolytic processing. One of the natural substrates is the viral assembly protein, which in the case of HSV-1 is known as ICP35. ICP35 is critical for the construction of intermediate viral capsids within the infected cell nucleus, and it is processed by the viral protease prior to DNA entry into the capsids. The other natural substrate is the protease precursor protein. The HSV-1 protease catalytic domain is localized in the N terminus of the precursor, which encompasses the N-terminal 247 amino acids of the 635-amino acid precursor protein (9–11). The herpesvirus proteases have been classified as serine proteases based on chemical reactivity toward classical serine protease inhibitors and site-directed mutagenesis data (12, 13). The catalytic efficiency of the HSV-1 protease is orders of magnitude less than expected of classical serine proteases, and no amino acid sequence homology has been found with them (14).

Recently, we and others have reported seemingly different activation phenomena for the HSV-1 and the hCMV proteases (15–18). In the case of the HSV-1 protease, antichaotropic cosolvents (also known as kosmotropes) increase the specific activity as much as 200-fold. Accompanying the activity changes are spectral changes of the protein indicating an altered conformation or aggregation state (15, 16). On a molar basis, the most effective activator found is citrate, followed by phosphate. For the hCMV protease, marked activity increase is observed with the use of glycerol (5). It is now known that the hCMV protease is only active when in a dimeric state, that the dissociation constant is in the micromolar range, and that glycerol or high enzyme concentrations favor the dimer formation (17, 18). This report identifies the activation of HSV-1 protease as similar to that of the hCMV protease with the demonstration that the active form of the HSV-1 protease is a dimer and that the activating antichaotropes promote the dimeric state. The unification of HSV-1 and hCMV protease activation phenomena as dimerization suggest that it is a common mechanism for the proteases of the herpes family of viruses. Aggregation as a prerequisite of activity may be more common than previously appreciated for the serine proteases.

MATERIALS AND METHODS

Enzyme Expression and Purification—The expression and purification of HSV-1 protease was performed as described previously (19), with the purification protocol modifications as later described (16).

Kinetic Assays and Equilibrium Constants—Assays were performed with the peptide substrate AGHTYLQASEKFKMWG, which HSV-1 protease cleaves between alanine and serine. The substrate was obtained from Bachem (Torrance, CA) and was used at 158 μM in all assays. The product SEKFKMWG was quantified on HPLC using fluorescence detection of the tryptophan residue. The buffer common to all assays, preincubations, and chromatography consisted of 52 mM MES, 52 mM TAPSO, 100 mM diethanolamine, 1 mM EDTA, 1 mM dithiothreitol, pH 7.5. Sodium phosphate or sodium citrate were included in some assays as indicated in the text. Activity assays were reactions of 60 s and were quenched with urea to a final concentration of 3 μl. For all solvent conditions used in specific activity measurements, the enzymic reaction progress curves were shown to be linear for at least 2 min. The dimer dissociation constants (Kd) derived from kinetic measurements were from fits of the equation

\[
\frac{v_{obs}}{v_{act}} = \frac{[E]}{[E] + [M]} \frac{1}{[E]},
\]

(Eq. 1)
was present at 0.2 M (300 mm) under all conditions. Repeated measurements of the enzyme that had been equilibrated for 3 h at 15 °C. Sodium phosphate and the original sample was injected again to confirm the constancy of background. Following a fractionation, the monitor was reconnected, 30-min assay to achieve product levels at least 10-fold higher than the accumulated product was quantified on HPLC. The dilution of the enzyme to ensure that equilibrium was achieved. In general, Kd measurements were unchanged after 3 h of equilibration under all conditions.

Size-exclusion Chromatography—A Pharmacia Superdex 75 column (300 × 10 mm) was used immersed in a controlled temperature water bath and connected to a manual injection valve immersed in the same bath with only the injection port exposed. Chromatography was at 5 °C in pH 7.5 buffer containing 20% glycerol and 0.4 M sodium citrate, pH 7.5. Sample volumes injected were 100 µl. Detection of eluting HSV-1 protease was by monitoring of absorbance (280 nm) and fluorescence (excitation 280 nm, emission 350 nm). The peak shapes and sizes obtained were unchanged for samples preincubated at 15 °C from 1 to 8 h. Column buffers were degassed with and maintained under helium. Proteins used for molecular mass standardization were bovine serum albumin (64 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa). When eluting HSV-1 protease peaks were collected for assay, the column effluent was not directed through the spectrometer monitors in order to avoid warming of the samples. Fractions were manually collected in ice-chilled tubes according to predetermined elution times. A small volume of substrate was added to initiate the activity assay. After 30 min, the assay was quenched, and the accumulated product was quantified on HPLC. The dilution of enzyme that occurs during chromatography necessitated the use of a 30-min assay to achieve product levels at least 10-fold higher than background. Following a fractionation, the monitor was reconnected, and the original sample was injected again to confirm the constancy of the elution times.

RESULTS

Enzyme Concentration Effects upon HSV-1 Protease Activity—The specific activity of HSV-1 protease was dependent upon enzyme concentration, as shown in Fig. 1. The data shown are indicative of a protomer-oligomer equilibrium in which the oligomer was more active. To assess the rate at which this equilibrium was established, activity as a function of time after a large dilution was examined under a variety of solvent conditions and temperatures. As exemplified by the data shown in Fig. 2, HSV-1 protease activity declined following a dilution of the enzyme from 10 µM to 100 nM. At 5 °C the activity was most stable, while at 15 °C and 20 °C, an approach to a new lower level of activity was apparent within minutes and was nearly complete at 2 h. These kinetic profiles of activity subsequent to dilution were consistent with the notion of a more active oligomer slowly dissociating to less active protomers.

Size-exclusion Chromatography—Size-exclusion chromatography was employed to determine the aggregation states of HSV-1 protease that might contribute to the kinetic behavior described above. The choice of conditions for the chromatography was aided by the stability data shown in Fig. 2, which indicate that a low temperature such as 5 °C was needed to minimize re-equilibration of enzyme forms during chromatography. Size-exclusion chromatography at 5 °C of an HSV-1 protease sample that was pre-equilibrated for 3 h at 15 °C revealed 2 different size species, as shown in Fig. 3. The elution volumes of the two peaks labeled b and a in Fig. 3A correspond to molecular weights of 21,000 and 55,000, respectively. Given the theoretical molecular weight of 27,065 for the 247-amino acid protein, these peaks correspond to monomer and dimer forms of the enzyme. We noted that resolution of these peaks was dependent upon the temperature and solvent used for the column, so that higher temperatures or the omission of citrate from the chromatography buffer produced elution patterns where the dimer peak merges into the monomer (data not shown).

When the enzyme was incubated prior to chromatography in buffer lacking glycerol or citrate, it was predominantly monomeric, as shown in Fig. 3A. Inclusion of 20% glycerol in the enzyme sample during incubation induced the formation of a small proportion of dimer (peak a). The activating anions phosphate and citrate gave increasing proportions of dimer, as shown in Fig. 3. Citrate was more effective than phosphate in shifting the equilibrium toward dimer.

The eluted peaks from size-exclusion chromatography were

2 The column used successfully for our previous study of hCMV protease aggregation state (17), a silica-based BioSelect 125 from Bio-Rad, produced elution profiles with asymmetric peaks and anomalous retention times with the HSV-1 protease, indicating interaction of the protein with the column matrix. No such effects were ever observed with the dextran-based Superdex media used here.
assayed for HSV-1 protease activity. The results are shown in Fig. 4. The dimeric enzyme (fractions a1 and a2) was more active than the monomer (b1 and b2). In addition, the lack of baseline resolution between monomer and dimer means that there was some dimer contribution to the activity observed in fractions b1 and b2, and that the intrinsic activity of the monomer was even less than that shown in Fig. 4.

**Kd Determinations**—Given the physical demonstration of a monomer-dimer equilibrium and the large difference in observed activity for the two forms of the enzyme, a kinetic model can be proposed to describe the dependence of specific activity upon enzyme concentration. In the model, the monomeric enzyme is inactive, and all activity is due to dimers. Hence, the concentration of active dimers is a function of the total enzyme concentration and the dimer dissociation constant $K_d$ (see “Materials and Methods” and “Discussion”). Data for the enzyme concentration dependence of specific activity are well represented by this model, as can be seen by the satisfying fit of the model to the data in Fig. 1. Different solvent conditions were examined for effect on the $K_d$, with the experimental approach illustrated in Fig. 1. In a buffer containing 20% glycerol without phosphate or citrate, the $K_d$ is too high to measure ($>1600 \text{ nM}$). Addition of phosphate or citrate to 0.2 M sodium citrate before 3 injections of 100 $\mu$l onto the column. The first injection established the elution pattern and the times at which to collect fractions, the second injection was used for fraction collection and assay, and a third injection was performed to ensure the continuous reproducibility of the elution pattern. The third elution profile was indistinguishable from the first. A, the elution pattern obtained, with the fractions collected labeled. B, the relative specific activity of the fractions collected. The values shown are normalized to the most active fraction (100%).

**DISCUSSION**

Initial reports of antichaotrope (kosmotrope) activation of the HSV-1 protease suggested that a change in the physical state of the enzyme, as indicated by spectral changes, is coincident with activation (15, 16). Here we present kinetic and physical data consistent with dimerization being the activating physical event.

Size-exclusion chromatography demonstrates the existence of two forms of the HSV-1 protease, identified as monomer and dimer on the basis of apparent molecular weights (Fig. 3). The more facile re-equilibration of HSV-1 protease monomer and dimer forms compared to the hCMV protease. Size-exclusion chromatography performed with solvent conditions used previously for the hCMV protease (10 °C, 20% glycerol, Ref. 17) produces a broad peak, not resolving the monomer and dimer forms (data not shown). A temperature of 5 °C and the addition of 0.4 M sodium citrate to the column buffer were
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15 °C for 60 s as described under "Materials and Methods." The enzyme was then diluted 1:10 in buffer with 20% glycerol and the salts indicated. Assays were at 0°C for 0.25, 0.30, and 0.35 ml min⁻¹ necessary to observe resolution of the HSV-1 protease monomer and dimer. Despite these additional measures to slow re-equilibration of an injected sample, column flow rates of 0.25, 0.30, and 0.35 ml min⁻¹ produce slightly different monomer-dimer ratios for a single sample, indicating that some re-equilibration of enzyme forms occurs during the chromatography (data not shown). In contrast to the previous study of hCMV protease, therefore, quantitative comparison of kinetic and chromatographic $K_d$ determinations is not possible. Nonetheless, size-exclusion chromatography used here as a qualitative measure of dimerization provides data completely consistent with interpretation of the kinetics.

The use of low temperature for chromatography, fraction collection, and assay enabled the direct demonstration that the dimer is the active form of the HSV-1 protease (Fig. 4). The temperature of 0°C for assays of the eluted fractions minimizes possible monomer-dimer re-equilibration during assays, although the 30-min assay time required to observe significant activity of the dilute fractions may have allowed substrate-induced dimerization to occur during the reaction, contributing to the activity seen in the monomer fractions. Substrate-induced dimerization is known to occur with hCMV protease (17). Additional activity in the monomer peak is contributed by a dimer not fully resolved from the monomer peak.

A simple model to describe the activity of the HSV-1 protease is depicted in Scheme 1 below.

$$K_d \quad M + M \rightleftharpoons D$$

$$D + S \rightleftharpoons D \cdot S \rightleftharpoons D + P_1 + P_2$$

$$K_{cat} \quad k_{cat} / K_m \quad D + S \rightleftharpoons D + P_1 + P_2$$

**Scheme 1**

Catalytic activity arises solely from dimeric enzyme in this scheme. While we cannot rule out a very low level of monomeric enzyme catalytic activity, the data are consistent, with the monomer contribution being negligible. Use of this scheme for the kinetic determination of dissociation constants $K_d$ under a range of conditions demonstrates the correlation of dimer formation with activation by antichaetropes (Table I). The parallel increases in dimer proportion seen with size-exclusion chromatography complete a self-consistent body of evidence for antichaetropes.

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