Communication

Activation of the Herpes Simplex Virus Type 1 Protease*

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Dawn L. Hall and Paul L. Darke†

From the Department of Biological Chemistry, Merck Research Laboratories, West Point, Pennsylvania 19486

The catalytic efficiency of the mature HSV-1 protease has been examined as a function of solvent composition. With the peptide substrate HTYLQASEKFKMW-amide, the specificity constant (kcat/Km) at pH 7.5 for cleavage is 5.2 M-1 s-1. This value increases to 38 M-1 s-1 when 25% glycerol is present in the reaction mixture. It was found that glycerol activation is but one case of the general phenomenon of HSV-1 protease activation by kosmotropes, or water structure-forming cosolvents. For example, an 860-fold increase in the protease activity (kcat/Km = 4500 M-1 s-1) occurs in the presence of 0.8 M sodium citrate. Similarly, the presence of 0.8 M sodium phosphate activates the catalytic efficiency by 420-fold (kcat/Km = 2200 M-1 s-1). The extent of HSV-1 protease activation by various anions correlates with the Hofmeister series. Both the susceptibility to proteolysis by trypsin and the protein fluorescence spectra of the HSV-1 protease change in the presence of activating solvents, suggesting a conformational change accompanying activation.

The catalytic activity of the herpes simplex virus type 1 (HSV-1)1 protease is essential for viral nucleocapsid formation and for viral replication (1, 2). The proteases of the herpesviruses are synthesized as precursor proteins that undergo autoproteolytic processing during viral assembly. The protease catalytic domain is localized in the N terminus of the precursor, which in the case of HSV is the N-terminal 247 amino acids of the 635-amino acid precursor protein (3–5). The natural substrates for the HSV protease are the viral protease precursor and the viral assembly protein known as VP22a or ICP35 (infected cell protein 35). The protease precursor and ICP35 are encoded by the U126 and U126.5 genes of HSV-1, respectively. The open reading frames of these genes overlap such that the smaller open reading frame of U126.5 is identical to the C-terminal end of the open reading frame of U126, the protease precursor gene (6, 7).

ICP35 is present in an immature form of HSV capsids, known as B capsids, during capsid assembly within infected cell nuclei. The proteolytic conversion of ICP35 from this immature form (ICP35icd) to the shorter form found only within cell nuclei (ICP35ef) is concomitant with the conversion of B capsids to capsids that contain viral DNA, known as C capsids. Thus, HSV protease action occurs within the cell nucleus and possibly within the viral capsid itself during a morphological transformation of capsids (8).

The necessity of HSV protease activity for the infectivity of HSV has prompted recent in vitro characterization of this enzyme and the related protease from human cytomegalovirus (HCMV) (9–13). Inactivation with disopropyl fluorophosphate was used to identify these enzymes as serine proteases (10), but sequence analyses have not revealed obvious homologies with the well-characterized groups of serine or other proteases. Peptides containing the sequences found in natural protein substrates are cleaved between a characteristic Ala-Ser sequence. Using peptide substrates, we have reported a kcat/Km for the purified mature HSV protease of 38 M-1 s-1, and DiBlasi et al. reported a kcat/Km of 37 M-1 s-1 with a similar assay condition that included 25% glycerol (9, 12). These values are many orders of magnitude lower than found for other serine proteases such as chymotrypsin and thrombin (107 M-1 s-1) (14, 15) and much lower than observed for other viral proteases such as rhinovirus 3C protease (1440 M-1 s-1) (16) and human immunodeficiency virus protease (13,000 M-1 s-1) (17). While it is conceivable that the low catalytic efficiency of the HSV protease observed in vitro may be sufficient to account for its essential physiological role in nucleocapsid assembly, the low activity prompts the consideration that some factor might enhance catalytic efficiency, such as an accessory cellular or viral component or appropriate solvent conditions. This report describes our findings of a large activation effect of kosmotropes, or water structure-forming substances, on the catalytic efficiency of the HSV-1 protease. The results suggest that a different conformation of the enzyme in the presence of kosmotropes is a factor affecting its action in catalyzing amide hydrolysis.

MATERIALS AND METHODS

Bacterial Expression and Enzyme Purification—A 306-amino acid precursor form of the HSV-1 protease was expressed in Escherichia coli cells and purified with a modified version of the protocol described previously (12). Briefly, soluble enzyme precursor from lysed E. coli cells was chromatographed on Fractogel-SO3 (Merck) at pH 7.0 with elution by a gradient of sodium citrate. Autoprocessing to the mature 246-amino acid form of the enzyme was achieved by the addition of 1.8 M sodium citrate to peak fractions to a final concentration of 0.5 M, followed by incubation at 4 °C for 2 h. The processed sample was desalted on Sephadex G-25 (Pharmacia Biotech Inc.), resolved on RPHPLC on Fractogel-SO3 affording a preparation of the mature enzyme that was >95% pure as judged by SDS-PAGE with Coomassie Brilliant Blue staining. N-terminal sequencing demonstrated that the N terminus began with alanine (codon 2), such that the first encoded amino acid, methionine, had been removed. Thus, the protein is one amino acid shorter (246) than the encoded length (247 amino acids). Protein concentrations were determined by quantitative amino acid analysis.

Cosolvent Effects—For experiments examining anion effects, buffer solutions were prepared with the sodium salt of the anion, and the pH was adjusted to 7.5 with concentrated HCl where necessary (i.e. citrate, glutamate, and malate). An exception was phosphate, where Na2HPO4 was prepared in the buffer, and the pH was adjusted upward with NaOH.

Activity Measurements—Enzyme activity was measured with the peptide substrates HTYQASEKFKMW-amide and HTYQASEKFWMW-amide using high pressure liquid chromatography for the separation of products from substrates and for quantitation (12). C-terminal cleavage product detection was with a Hewlett-Packard Model 1046A fluorescence detector (excitation at 280 nm and emission at 350 nm),
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RESULTS

Activation by Anions—The HSV-1 protease had a specificity constant ($k_{cat}/K_m$) of $5.2 \text{ M}^{-1} \text{s}^{-1}$ at $37^\circ \text{C}$ in pH 7.5 with no glycerol present using a peptide substrate that represents the cleavage site that generates the mature protease C terminus, HYTLYQASEKFKMW-amide. Under initial velocity conditions, where the reaction velocity was shown to be constant for 60 min, the rate was linearly proportional to substrate concentration from 0.1 to 2 mM. Thus, the $k_{cat}$ was well above the highest substrate concentration tested, and $k_{cat}/K_m$ was calculated from the slope of the velocity-concentration line.

The effect of various salts on activity was examined in assays in which the HSV-1 protease and substrate concentrations were maintained constant. As shown in Table I, many salts increased the activity of the protease, particularly those with multivalent anions. In the case of sodium citrate at 0.8 M, for instance, the observed hydrolytic rate was enhanced 202-fold. Phosphate and sulfate were also potent activators of the protease, increasing activity 118- and 110-fold, respectively. Chloride had little effect, while bromide, iodide, and perchlorate were inhibitory. The order of anion effectiveness for activation on a molarity basis (as opposed to ionic strength) is $\text{Br}^- < \text{Cl}^- < \text{CH}_3\text{C}_\text{O}^- < \text{F}^- < \text{SO}_4^{2-}, \text{PO}_4^{3-} < \text{citrate}^3$. This is the same order as the Hofmeister series of anions (18). The activation of the HSV-1 protease by anions was insensitive to stereoechemical configuration, as evidenced when isoctirate was compared with citrate or when L-malate and L-glutamate were compared with their D-isomers (Table I). The effect of the counters on HSV protease activity was relatively insignificant, with (NH$_4$)$_2$SO$_4$ being slightly less activating relative to the Na$^+$ and K$^+$ sulfate salts (data not shown).

Activation was also observed with the multiply hydroxylated alcohols, glycerol, and sorbitol, as shown in Table II. Other cosolvents of more hydrophobic character, such as ethanol and dimethylformamide, were strongly inactivating, even at 2%. We note, however, that inhibition by these solvents could be overcome by the activating cosolvents. For example, while 2% dimethyl sulfoxide reduced HSV protease activity 37% (Table II), it only reduced the activity 8% with 0.8 M sodium citrate present (data not shown).

The stability of the protease activity in the presence of various additives was examined. In all cases, including no additive, 0.8 M citrate, 10% glycerol, or 0.2 M bromide, the accumulation of product was linear over a 60-min period. Hence, the wide differences in enzyme activity seen when various salts were added were not due to time-dependent differences of protease stability in the assays.

The activation of the HSV-1 protease by increasing citrate and phosphate concentrations was progressive and did not saturate at the highest salt concentration tested, as shown in Fig. 1. The greatest effects for either citrate or phosphate appeared at concentrations of 0.4 M and above, although some activation was also observed at a concentration as low as 0.05 M (Fig. 1, inset). In the absence of activators, substrate saturation was observable within the limits of peptide solubility, so $k_{cat}$ and $K_m$ parameters in the presence of 0.2 M sodium citrate were determined to be 3 min$^{-1}$ and 1.32 mM ($k_{cat}/K_m = 40 \text{ M}^{-1} \text{s}^{-1}$), respectively, and those in the presence of 0.8 M citrate were determined to be 4 min$^{-1}$ and 0.016 M ($k_{cat}/K_m = 450 \text{ M}^{-1} \text{s}^{-1}$), respectively. Relative to the case where no activator is present ($k_{cat}/K_m = 5.2 \text{ M}^{-1} \text{s}^{-1}$), $k_{cat}/K_m$ increased 860-fold in the presence of 0.8 M citrate. Similarly, using a peptide substrate representing the catalytic site within ICP35, ALVNAS-SAAHVDV-amide, $k_{cat}/K_m$ increased from 0.45 to 488 M$^{-1} \text{s}^{-1}$ (1085-fold) by changing citrate from 0 to 0.8 M, demonstrating that activation is not substrate-specific.

Nucleotide Effects on Activity—Nucleotides and polynucleotides were examined for possible effects on HSV protease activity. The results are shown in Table III. While some activation can be seen with triphosphate nucleotides such as CTP and dGTP, other nucleotides were inhibitory. The concentrations of polynucleotides examined were limited due to solubility. Within the range of polynucleotide concentrations examined, the effects are minor in comparison with those seen with much higher concentrations of anions (Table I).

Anion Effects on the HSV-1 Protease Alone—Fluorescence emission spectra of the HSV-1 protease were shown in Fig. 2A. The emission maximum is 354 nm in the absence of any activators. In the presence of citrate, the emission spectra shift toward shorter wavelength maxima with markedly greater intensity (Fig. 2). Phosphate was also observed to produce a similar shift (data not shown). In contrast, the same solvent changes produced a red shift in emission maximum for N-acetylcysteine, as shown in Fig. 2B. Short peptides
containing tryptophan also exhibited a red shift in the higher salt buffers (data not shown). Titration of wavelength shifts and intensity changes as a function of citrate concentration produced minimal effects on the HSV-1 protease at concentrations ≤ 0.4 M, but were much more pronounced at higher concentrations, as shown in Fig. 2.

**DISCUSSION**  
In this work, we have described solutes that enhance the specificity constant for the HSV-1 protease ($k_{cat}/K_m$) orders of magnitude over that found in simple aqueous buffers. The $k_{cat}/K_m$ of 4500 M$^{-1}$ s$^{-1}$ reported here in activity assays containing 0.8 M sodium citrate is ~860 times greater than in the absence of citrate (5.2 M$^{-1}$ s$^{-1}$) using the substrate HTYLQASEKFKMW-amide. We have previously reported a specificity constant of 38 M$^{-1}$ s$^{-1}$ using the same substrate in 25% glycerol (12), a cosolvent somewhat less activating than...
citrate. The specificity constants listed here for the HSV protease can be contrasted with that found for the closely related recombinant HCMV protease in the absence of any of activators (404 M$^{-1}$s$^{-1}$) (11). Some increase in the activity of the HCMV protease has been noted in the presence of glycerol (19), although the kinetics have not been examined in detail.

The anion activation data reported herein for the HSV protease are not characteristic of a site-specific binding event as no saturation of the activation was observed (Fig. 1). In addition, a site-specific activation would be expected to display chemical structure correlations, but in the examples shown here (Table I), phosphate, citrate, and isoctonite exhibited similar effects on activity. The observed order of potencies for anion activation of the HSV protease correlates with the Hofmeister series of anions, such that the effectiveness for activation is Br$^-$, I$^-$, Cl$^-$ < CH$_3$COO$^-$ < F$^-$. SO$_4^{2-}$ < citrate$^-$. HSV-1 protease activation is thus a general solvent effect. Indeed, the activation data described here have the characteristic Hofmeister effect attributes: the effects become apparent at moderate concentrations (0.01–1 M); the effects are dominated by anions; and there is a sign inversion of effect at NaCl (reviewed in Ref. 18). The anions producing activation of the HSV-1 protease are also those that have been termed kosmotropes, or water structure-forming solutes.

A comparison of the $k_{cat}$ and $K_m$ values for the substrate HTYLQASEKFKMW-amide using different sodium citrate concentrations revealed that the kinetic parameter most changed by the kosmotropes is the $K_m$, such that 1.32 and 0.016 mM were obtained as $K_m$ values for 0.2 and 0.8 M citrate, respectively. Since kosmotropes are also solutes that produce “salting-out” and aggregation effects for proteins, it is conceivable that the lowering of $K_m$ observed here is a result of anion destabilization of the free peptide substrate in solution, producing a relative stabilization of the enzyme-substrate complex and a lowering of the observed $K_m$ (20). While there may be some contribution of anion destabilization of the unbound substrate to the lowering of $K_m$, the loss of susceptibility of the HSV-1 protease to trypsin digestion in 0.8 M citrate and spectral changes evidence a conformational change in the HSV-1 protease (Figs. 2 and 3). Hence, kosmotropes may promote a conformational state of the mature HSV-1 protease with a greater affinity for substrate than exists in simple aqueous solution.

The activation described here raises important questions regarding the conditions under which protease catalysis occurs in vivo during virus assembly. Is the nucleus an activating microenvironment, with its abundance of nucleotide polyanions, so that the HSV protease is activated only after transport into the nucleus? Is the mature form of the HSV protease the biologically relevant form in terms of in vivo proteolytic events? Our examination of some likely nuclear activators did reveal activation by selected triphosphate nucleotides (Table III), but in view of the inhibition by other nucleotides, the meaning of these activity effects is unclear. It is important to note, however, that the DNA concentration in the vicinity of a developing capsid is much higher than we were able to test. In an HSV C-type capsid in which the DNA packaging is complete, the concentration of DNA phosphate diesters can be calculated to be $\sim 1.5$ M, or 450 mg/ml (21).

The proteases of the herpes group of viruses are synthesized as precursor proteins. In the case of the HSV protease, Jones et al. (22) have found that the HCMV protease precursors appear to be as active as the mature form of the HCMV protease in a bacterial expression system. It remains to be determined which of the protease forms of either HSV or HCMV, precursor or mature, actually perform the cleavages during capsid maturation. Since the mature HSV protease appears to have less activity that the mature HCMV protease in vitro assays containing no special additives (5.2 versus 404 M$^{-1}$ s$^{-1}$), it may be that the most important functional form of the HSV protease is an activated form, possibly a precursor. Kosmotropes induce in the mature HSV protease a more active conformation of the catalytic domain, possibly one occurring naturally when the domain is in association with other viral components. It remains for the precursor forms of the herpesvirus proteases to be isolated and evaluated as catalysts.

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