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Substrate Modulation of Enzyme Activity in the Herpesvirus Protease Family

Ana Lazic[1]†, David H. Goetz[1]†, Anson M. Nomura[1,2], Alan B. Marnett[1,2] and Charles S. Craik[1,2]*

[1]*Department of Pharmaceutical Chemistry, University of California, San Francisco CA 94158-2517, USA*

[2]*Graduate Program in Chemistry and Chemical Biology, University of California, San Francisco CA 94158-2517, USA*

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The herpesvirus proteases are an example in which allosteric regulation of an enzyme activity is achieved through the formation of quaternary structure. Here, we report a 1.7 Å resolution structure of Kaposi's sarcoma-associated herpesvirus protease in complex with a hexapeptide transition state analogue that stabilizes the dimeric state of the enzyme. Extended substrate binding sites are induced upon peptide binding. In particular, 104 Å^2 of surface are buried in the newly formed S4 pocket when tyrosine binds at this site. The peptide inhibitor also induces a rearrangement of residues that stabilizes the oxyanion hole and the dimer interface. Concomitant with the structural changes, an increase in catalytic efficiency of the enzyme results upon extended substrate binding. A nearly 20-fold increase in k_{cat}/K_M results upon extending the peptide substrate from a tetrapeptide to a hexapeptide exclusively due to a K_M effect. This suggests that the mechanism by which herpesvirus proteases achieve their high specificity is by using extended substrates to modulate both the structure and activity of the enzyme.

Keywords: allostery; induced fit; viral protease; protein-protein interactions; X-ray structure

Introduction

The herpesvirus family encodes a protease whose activity is regulated by a unique oligomerization dependent allosteric mechanism.[1–6] The inactive monomeric protease is expressed fused to an assembly protein in the cytosol and subsequently imported into the nucleus.[7–11] Concentration dependent dimerization within the nuclear localized immature capsids activates the protease.[1,2,12] The active protease is released from the assembly protein by auto-processing at the release (R) site.[10,11,13–15] Protease processing of the assembly protein, a major scaffold protein, at the maturation (M) site leads to capsid maturation and virion formation.[11,14,16,17]

Recent studies on the Kaposi's sarcoma-associated herpesvirus (KSHV) protease suggest an induced structure upon dimerization as a concentration dependent activation of the enzyme.[4–6] This induced structure model differs from canonical induced fit in that the free energy of subunit dimerization is used to organize the active site and provide a unique mechanism for activating proteolytic activity. In this model, dimerization induced folding of helix 5, the major contributor to the dimer interface, and helix 6, which is crucial for stabilization of the oxyanion hole, stabilizes elements that are essential for catalytic activity.

Similarly, structural studies of the related human cytomegalovirus (HCMV) protease,[3] reveal loss of catalytic activity of a dimer interface mutant due to disordering of both the C-terminal helix G and the L10 loop (H6 and L10 in KSHV protease) that support the catalytic machinery. In addition, the ordering of these structural elements, in the HCMV protease variant can be induced by binding of a peptidomimetic inhibitor. Finally, upon binding of a peptide phosphonate inhibitor at the active site of KSHV protease, the equilibrium is shifted toward

**Corresponding author.* E-mail address: craik@cgl.ucsf.edu.

† A.L. and D.H.G. contributed equally to this work.

Abbreviations used: KSHV, Kaposi's sarcoma herpesvirus; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; DFP, diisopropyl fluorophosphonate; R, release; M, maturation.

the dimeric enzyme, suggesting communication between the active site and the dimer interface.[4]

We sought to understand how substrate binding and its effect on the disorder-to-order transition could regulate catalytic activity in KSHV protease. To understand the structural basis of the stabilized oligomer state we have used the hexapeptide diphenylphosphonate transition state analog previously shown to dramatically shift the equilibrium to the dimeric form of the enzyme. We have crystallized KSHV protease bound to this inhibitor and determined the structure of the complex. The structure elucidates atomic details of substrate binding pockets, which are composed of loops previously disordered in the apo structure of KSHV protease. In addition, an induced fit to the extended substrate binding pockets of the protease is revealed, which is most pronounced at S4. The structural evidence for protease extended substrate specificity that leads to enhanced catalytic efficiency is revealed. This suggests that substrate binding could provide another level of regulation of protease activity in this enzyme beyond the initial concentration dependent activation mechanism described previously.

Results

Extended substrate binding improves catalytic efficiency

The positional scanning synthetic combinatorial libraries previously used to determine the KSHV non-prime side substrate specificity contained only tetrameric substrates.[4] To determine the extent of the extended substrate specificity for KSHV protease we synthesized five nested sets of substrates with the trimeric *t*butyl-Gln-Ala being the shortest and the heptameric Ser-Pro-Val-Tyr-*t*butyl-Gln-Ala substrate being the longest. All substrates were acetylated on their N termini and included the fluorogenic reporter group 7-amino-4-carbamoyl-methylcoumarin to monitor enzyme activity spectroscopically. The amino acid sequence of the tri-, tetra-, penta- and hexameric substrates corresponds to the sequence of the hexapeptidyl phosphonate inhibitor,[4] while in the heptapetide, Ser was incorporated at the P7 position in accordance with the natural R site sequence.

Kinetic analysis of the peptide substrates (Table 1) reveals an increase in catalytic efficiency for the hydrolysis of longer substrates with an 18.2-fold increase for heptameric compared to tetrameric substrates. The improvement in catalytic efficiency for longer substrates comes solely from improved K_M values, whereas the turnover rate, k_{cat} remains virtually unchanged.

Using the high-resolution three-dimensional structure of the enzyme inhibitor complex (see below) we calculate that binding of the hexapeptide inhibitor buries 450 Å^2 of hydrophobic surface area (Table 1). Binding of the tetrapeptide inhibitor would bury 360 Å^2. This difference of 90 Å^2 between hexapeptide and tetrapeptide binding contributes 1.44 kcal/mol to substrate binding energy. This is in agreement with the 1.40 kcal/mol difference in Gibbs free energy calculated for binding of these two substrates from their k_{cat}/K_M values.

Ordering of unstructured regions of KSHV protease

The crystal structure of the KSHV protease complexed with a hexapeptide transition state analogue was determined at a resolution of 1.7 Å (Figure 1(b)). The overall fold of the KSHV protease–inhibitor structure is similar to the reported apo protease structure[18] (Figure 1(b)). However, several previously disordered regions are present in our structure accounting for new secondary structure elements, namely, three loops and five helices (Figure 1(c)). The ordered loops L0a, L0b and L9 encompass the following residues: 15–20, 24–26, and 125–129, respectively. To preserve previous helix nomenclature we have kept the same numbering for the helices and we introduce a letter following the already assigned corresponding helix number to annotate new helices. Also, we annotated a small helix encompassing residues 88–91 that was left out from the nomenclature in the previous structure of apo KSHV protease. Thus, we define helices as follows: α0, residues 21–23; α0a, residues 27–33; α1, residues 74–84; α1a, residues 88–91; α2, residues 100–110; α2a, residues 120–124; α3, residues 154–159; α4, residues 166–179; α4a, residues 181–183; α5, residues 193–20; and α6, residues 209–220.

Newly ordered regions (Figure 1(c)) encompass residues involved in substrate binding that belong to the extended substrate binding pockets. The helix 2a residues Pro120, Arg121 and Glu122 contribute to the formation of the P3, P5 and P6 binding pockets

Table 1. Hydrolysis rates of the extended substrates for KSHV protease

		k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	Surface area (Å^2)	ΔΔG (kcal/mol)
P3-P1	Ac-tQA-ACC	N.D.	N.D.	N.D.		
P4-P1	Ac-YtQA-ACC	0.0084±0.0003	80.0±8.8	105	359	5.7
P5-P1	Ac-VYtQA-ACC	0.0075±0.0003	15.9±2.5	472	408	6.5
P6-P1	Ac-PVYtQA-ACC	0.0083±0.0002	8.5±0.8	976	446	7.1
P7-P1	Ac-SPVYtQA-ACC	0.0088±0.0003	4.6±0.7	1913	N/A	N/A

The surface area that substrate buries upon binding is calculated from the structure of complexed KSHV protease. The difference in Gibbs free energy for binding of the each residue was determined based on the burial of hydrophobic solvent accessible surface area of protease upon substrate binding. N.D., not determined.