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Insights into the serine protease mechanism based on structural observations of the conversion of a peptidyl serine protease inhibitor to a substrate

Longguang Jiang [a,b], Lisbeth Moreau Andersen [b,c], Peter A. Andreasen [b,c], Liqing Chen [d], Mingdong Huang [a,b,*]

[a] State Key Lab of Structural Chemistry, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, 155 Yang Qiao West Road, Fuzhou, Fujian 350002, China
[b] Danish–Chinese Centre for Proteases and Cancer, Denmark
[c] Department of Molecular Biology and Genetics, Aarhus University, Aarhus 8000-DK, Denmark
[d] University of Alabama in Huntsville, Huntsville, AL 35899, USA

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ABSTRACT

Background: Serine proteases are one of the most studied group of enzymes. Despite the extensive mechanistic studies, some crucial details remain controversial, for example, how the cleaved product is released in the catalysis reaction. A cyclic peptidyl inhibitor (CSWRGLENHRMC, upain-1) of a serine protease, urokinase-type plasminogen activator (uPA), was found to become a slow substrate and cleaved slowly upon the replacement of single residue (W3A).
Methods: By taking advantage of the unique property of this peptide, we report the high-resolution structures of uPA in complex with upain-1-W3A peptide at four different pH values by X-ray crystallography.
Results: In the structures obtained at low pH (pH 4.6 and 5.5), the cyclic peptide upain-1-W3A was found to be intact and remained in the active site of uPA. At 7.4, the scissile bond of the peptide was found cleaved, showing that the peptide became a uPA substrate. At pH 9.0, the C-terminal part of the substrate was no longer visible, and only the P1 residue occupying the S1 pocket was identified.
Conclusions: The analysis of these structures provides explanations why the upain-1-W3A is a slow substrate. In addition, we clearly identified the cleaved fragments of the peptide at both sides of the scissile bond in the active site of the enzyme, showing a slow release of the cleaved peptide.
General significance: This work indicates that the quick release of the cleaved P′ fragment after the first step of hydrolysis may not always be needed for the second hydrolysis.

1. Introduction

Serine proteases of the trypsin family (clan PA) play important physiological and pathophysiological roles. The human and mouse genomes contain 176 and 227 genes, respectively, encoding serine proteases with highly similar active sites [1]. Many serine proteases are potential therapeutic targets. Moreover, serine proteases are classical proteins for studying catalytic and inhibitory mechanisms [2,3]. The catalytic mechanism of serine proteases has been widely studied, and substantial biochemical and structural data have established a two-step mechanism for the hydrolysis of peptide bonds by serine proteases [2]. The first step of the reaction is a nucleophilic attack by the catalytic Ser195 on the carbonyl carbon atom of the P1 residue that generates a covalent acyl-enzyme intermediate and a new peptide amino terminus on the P1′ residue. A second nucleophilic attack by a water molecule leads to the hydrolysis of the acyl-enzyme intermediate, which releases the new carboxyl group and converts Ser195 back to its initial catalytically competent state.

Numerous crystallographic studies of serine proteases with bound substrates or inhibitors have provided detailed structural evidence regarding the intermediates involved in the catalysis, including the enzyme–substrate complex [4], the acyl-enzyme intermediate [5–9], and the high-energy tetrahedral intermediates [10–14]. However, there are still some critical issues in the mechanism which are not resolved yet. The current mechanism indicates that dissociation of the leaving group must occur prior to the second nucleophilic attack to give space for the water molecule to hydrolyze the acyl-enzyme intermediate.

Abbreviations: uPA, urokinase-type plasminogen activator; $uPA_{16–244}$, serine protease domain of uPA (residues 16–244); K_D, equilibrium dissociation constant; K_i, equilibrium inhibition constant; PAB, *p*-aminobenzamidine.

* Corresponding author at: State Key Lab of Structural Chemistry, Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, 155 Yang Qiao West Road, Fuzhou, Fujian 350002, China.
E-mail address: mhuang@fjirsm.ac.cn (M. Huang).
URL's: http://www.proteasesandcancer.org (L. Jiang), http://www.proteasesandcancer.org (L.M. Andersen), http://www.proteasesandcancer.org (P.A. Andreasen), http://www.proteasesandcancer.org (M. Huang).

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There is yet little evidence to support this [2]. Furthermore, the mechanism by which the P1 group of the substrate is released from the S1 pocket after peptide bond hydrolysis still remains largely elusive. Many previous studies focused on how specificity is generated when the substrate binds to the serine protease and how the peptide bond was hydrolyzed by the serine protease [15–17]. Usually, the affinity of substrate binding to the S1 pocket is high; thus, the other intriguing question is to determine how the P1 group is released from the S1 pocket after the peptide bond has been hydrolyzed by the serine protease.

To gain structural insights into the catalytic mechanisms of serine proteases, we determined high-resolution crystal structures of urokinase-type plasminogen activator (uPA) and a cyclic disulfide bridged peptide, a upain-1-W3A (CSARGLENHRMC) [18]. uPA is an important member of the serine protease family that catalyzes the conversion of plasminogen to its active form plasmin. uPA has been found to be a key mediator of several biological processes including fibrinolysis, angiogenesis, wound healing, tissue remodeling, tumor growth and metastasis [19]. uPA was chosen as the enzyme of study because the uPA crystal we generated has a special molecular packing with large open spaces around the enzyme active site. This uPA crystal form allows the soaking of small molecular or even peptidyl uPA inhibitors from solution directly into the uPA active site and provides a platform to measure the uPA-bound structures of different uPA inhibitors or substrates [20].

Upain-1-W3A was mutated from an uPA peptidyl inhibitor, upain-1 (CSWRGLENHRMC), which was previously identified from a phage-display peptide library [18]. The crystal structure of the uPA:upain-1 complex showed that the cyclic peptide has a distinct characteristic as an inhibitory-binder. Arg4 of upain-1 binds to the uPA S1 subsite. Another conspicuous observation refers to the P2 residue, Trp3, which does not occupy the S2 subsite, but instead binds in the uPA S4 subsite [20]. Upain-1 contains two potentially scissile peptide bonds (Arg4-Gly5 and Arg10-Met11). However, it behaves as an inhibitor of uPA, rather than a substrate. Interestingly, the substitution of the P2 residue Trp3 to Ala (upain-1-W3A) converts upain-1 from an inhibitor to a slow uPA substrate with scissile bonds located between residues Arg4 and Gly5 [18]. This slow substrate provides a tool to study the structural transition associated with the hydrolysis.

In this study, we determined crystal structures of uPA in complex with upain-1-W3A at four different pH values to high resolutions (1.29–1.68 Å), and these structures provide structural insight into the conversion of a peptidyl uPA inhibitor to a cleavable substrate upon pH change. At low pH (pH 4.6 and pH 5.5), the peptide remains intact, and all of the peptidyl residues are clearly defined in the structure. In contrast, at pH 7.4, the scissile bond between Arg4 and Gly5 of the peptide is cleaved, and both the P1–P2 and P1′–P2′ fragments are defined in the structure. At pH 9.0, the P1′–P2′ fragment is no longer visible, and only the P1 residue is defined in the S1 pocket. According to these observations, the release of the substrate, P1′–P2′ fragment and the P1 residue, can be one of the rate-limiting events of the hydrolysis.

2. Materials and methods

2.1. Materials

Para-aminobenzamidine (PAB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The chromogenic substrate Glu-Gly-Arg-*p*-nitroanilide (BIOPHEN CS-61(44)) was purchased from Aniara Diagnostica (Ohio, USA) and human two-chain uPA was purchased from ProSpec-Tany TechnoGene Ltd. (Israel). Synthesis of the cyclic constrained peptides upain-1 (CSWRGLENHRMC) and upain-1-W3A (CSARGLENHRMC) was carried out as described previously [21].

2.2. Expression and purification of recombinant uPA

Expression and purification of recombinant $uPA_{16–244}$ were described previously by Zhao et al. [20]. Briefly, $uPA_{16–244}$ was secreted from a stably transfected *Pichia pastoris* strain (X-33) after induction with methanol. A purified product was obtained after cation exchange chromatography (SP Sepharose Fast Flow from GE) followed by gel filtration chromatography using a Superdex 75 HR 10/300 column (GE) equilibrated with 20 mM phosphate buffer (pH 6.5) containing 150 mM NaCl. The protein was eluted as a single peak under these conditions with a retention volume of approximately 13.6 ml, corresponding to its molecular weight (28 kDa). The protein was dialyzed in 20 mM potassium phosphate (pH 6.5) overnight and concentrated to 10 mg/ml using stirred ultra-filtration cells (Millipore and Amicon Bioseparations, Model-5124).

2.3. Crystallization and data collection of uPA:upain-1-W3A complexes

The crystallization trials were carried out using the sitting drop vapor diffusion method. The crystals were obtained by equilibrating against a reservoir solution containing 50 mM sodium citrate (pH 4.6) and 2.0 M ammonium sulfate supplemented with 5% PEG400 at room temperature. The crystals, which appeared after approximately three days, were soaked for 72 h in crystallization mother liquor containing 1 mM upain-1-W3A. X-ray diffraction data of the uPA:upain-1-W3A complex were collected using synchrotron radiation at the Advanced Photon Source SER-CAT beam line 22-ID (Chicago, USA, Argonne National Laboratory). Prior to X-ray diffraction data collection, the crystals were soaked in one of the following cryoprotectant solutions at four different pH values for approximately 5 min: (A) 50 mM sodium citrate (pH 4.6), 2.0 M ammonium sulfate, 5% PEG400, and 20% glycerol; (B) 100 mM imidazole-malate (pH 5.5), 2.0 M ammonium sulfate, 5% PEG400, and 20% glycerol; (C) 100 mM Tris–HCl (pH 7.4), 2.0 M ammonium sulfate, 5% PEG400, and 20% glycerol; or (D) 100 mM Tris–HCl (pH 9.0), 2.0 M ammonium sulfate, 5% PEG400, and 20% glycerol.

X-ray diffraction data of each frozen crystal were collected at 100 K and processed using the HKL-2000 program suite [22]. All of the crystals belonged to the R3 space group with a single uPA:upain-1-W3A complex in the crystallographic asymmetric unit. The crystal structures of the uPA:upain-1-W3A complexes were solved by molecular replacement with *MORLEP* [23] using a uPA serine protease structure (PDB code: 2NWN) [20] as a search model. The models were further refined with *REFMAC* [24]. For structures of the uPA:upain-1-W3A at low pH, the F_o–F_c electron density maps calculated using the positioned molecular replacement model clearly demonstrated continuous F_o–F_c positive density at the binding site (Fig. S2A–B) and the upain-1-W3A was then built into the maps. At higher pH, the uPA:upain-1-W3A structures showed some discontinuous density in the active site. Such electron density maps are shown in the Fig. S2C–D, and they are likely less model biased as no any peptides were included in the model. The final model and the electron density maps were shown in Figs. 1, 3B and E. Due to the high resolution of all these structures (1.29–1.68 Å), water molecules were added into the electron density of the F_o–F_c map with an intensity of at least 2.5 σ using the molecular graphics program COOT [25]. We also determined the crystal structure of inactive uPA (uPA-S195A) in complex with upain-1-W3A at pH 7.4 (Fig. S2E) with the BL17U beamline at the Shanghai Synchrotron Radiation Facility (SSRF). Statistics from the data collection and the final model refinement for all these structures are summarized in Table 1. The final structures were analyzed by PyMOL [26].

2.4. Determination of the inhibition constant (K_i) for the inhibition of uPA by upain-1-W3A

The concentrations of the peptides were determined by measuring the OD_{280} and using the sequence-derived extinction coefficients provided by the Protparam tool provided by the ExPASy server (http://www.expasy.org). For routine determinations of K_i values for the inhibition of uPA under steady-state inhibition conditions, a fixed concentration of uPA (2 nM) was preincubated in 200 μl of HEPES-buffered saline