Structure of Thrombin Inhibited by Aeruginosin 298-A from a Blue-Green Alga

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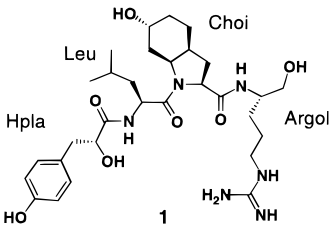
Hirudin is a natural thrombin inhibitor that occurs in medicinal leeches.[1] Its potency (K_i = 22 fM) comes from binding at the active site of thrombin and to the fibrinogen recognition exosite[2] that imparts additional selectivity to fibrinogen cleavage.[3,4] The C-terminal of hirudin associates with the exosite and can be mimicked by the C-terminal fragment hirudin 53−64 (hirugen). Aeruginosin 298-A (298-A) is a peptidic active-site protease inhibitor containing nonstandard amino acids (**1**) produced by a blue-green alga.[5,6] We report here the X-ray crystallographic



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structure of the ternary complex of 298-A bound to hirugen−thrombin at 2.1 Å resolution, which reveals several unexpected interactions that may be useful and of importance for structure-based drug design (Figure 1). The peptide chain of 298-A forms an antiparallel β-strand with thrombin similar to substrates. The guanidinium group of the Argol residue is in the primary S1 specificity site making a doubly hydrogen bonded salt bridge with Asp189, while the hydroxyl group of the Argol hydrogen bonds with His 57 disrupting the original hydrogen bonding system of the catalytic site triad (His 57, Ser 195, Asp 102). The Choi residue binds to the S2 peptide binding subsite of thrombin in the normal way. The Leu and Hpla residues at the P3 and P4 positions interact with thrombin in a new and unique way: L-Leu orients to partially occupy the hydrophobic D enantiomorphic S3 binding subsite even though it is a L enantiomer while Hpla follows the track of a L enantiomer at P3 even though it is the P4 residue (Figure 2).

Thrombin is the most selective and multifunctional of the enzymes that belong to a subclass of serine proteases that play vital roles in hemeostasis and thrombosis.[7−10] Its zymogen prothrombin (71.6 kDa) is cleaved by the plasma prothrombinase complex (factor Xa, factor Va, Ca^{+2}, and membrane phospholipid) to produce the highly active regulatory enzyme thrombin (39 kDa). In the complex cascade of coagulation, thrombin participates in a number of biochemical reactions, the most notable of which is the conversion of fibrinogen to fibrin,[3,4] which is readily converted to a cross-linked network that, with trapped platelets, is the blood clot. Thrombin has also been implicated in some events at the cellular level such as the activation of blood platelets[11,12] and in a number of other processes.[13−16] Because of its remarkable variety of functions in hemeostasis, thrombin has become a special target for molecular drug design. Thus far, however, no small to midsize drugs are used clinically to alter thrombin clotting properties.

Natural products, along with their complex variety of structural and modulatory biological effects, have provided rational insights regarding the design and development of useful drugs. However, only a small number of thrombin inhibitors of natural origin are known. Examples are cyclotheonamides A and B (from the marine sponge *Theonella* sp.),[17,18] bathojaracin (from snake venoms),[19] orthinodorin (from ticks),[20] hirudin,[1,2] and heparin.[21,22] During the course of screening protease inhibitors from marine natural sources, the aeruginosin family of highly active inhibitors was discovered produced by the fresh blue-green alga *Microcystis aeruginosa*, which present selective properties toward serine proteases.[5,6] One, 298-A, displays thrombin and trypsin inhibition (IC_{50} of 0.5 and 1.7 μM, respectively) but does not significantly inhibit papain, chymotrypsin, elastase, or plasmin.

Crystals of the ternary complex were prepared by repetitive diffusion of solutions containing increasing concentrations of 298-A into a crystal (0.15 × 0.20 × 0.25 mm^3) of hirugen−thrombin grown by the vapor-diffusion hanging-drop method.[23] The drop contained 1 μL of hirugen−thrombin (3.5 mg/mL) plus 1 μL of the well solution (0.1 M sodium phosphate buffer pH 7.3, 1 mM sodium azide and 25% (w/v) polyethylene glycol-8000). The crystal was soaked in 2 and 5 mM solutions of 298-A for 6 h each and finally for 5 days at 8 mM prior to X-ray data collection. Thrombin was initially complexed with hirugen at the fibrinogen recognition exosite to prevent autolysis during crystallization and subsequent inhibitor diffusion experiments.

The ternary complex is monoclinic, space group *C*2, a = 71.97, b = 72.48, c = 72.24 Å, β = 100.93°, and is isomorphous with crystals of hirugen−thrombin.[23] Room temperature intensity data were collected using a Rigaku RU200 rotating anode source equipped with a RAXISII imaging plate detector and processed

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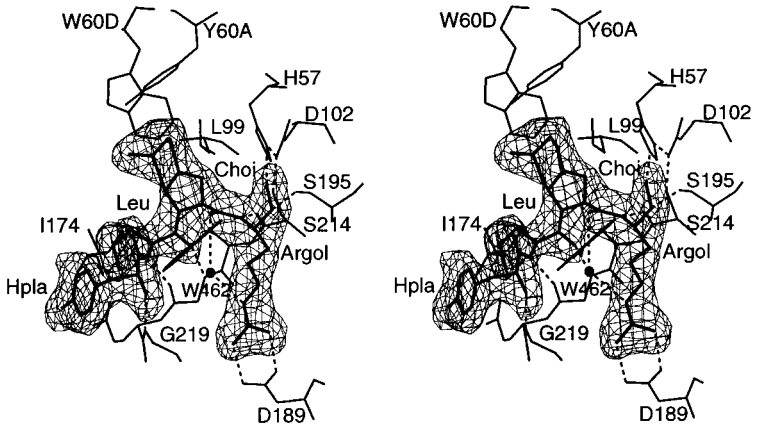

Figure 1. Stereoview of the binding of aeruginosin 298-A in the hirugen−thrombin complex. OMIT electron density of the final aeruginosin structure contoured at 2σ. Hydrogen bonds shown as broken lines; close Ser195OG-argininol carbon contact (2.8 Å) also broken line.

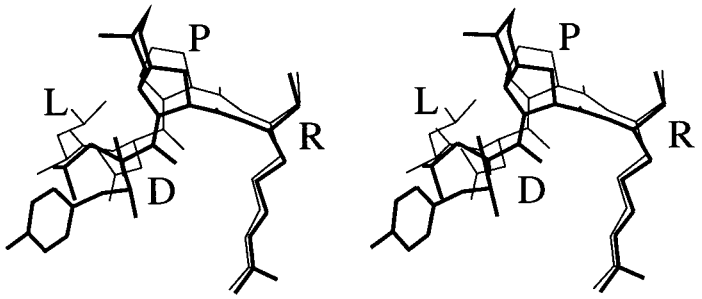

Figure 2. Stereoview of the superposition of the bound thrombin structures of aeruginosin 298-A (bold) and the Leu-Asp-Pro-Arg peptide of thrombin platelet receptor (thin lines).[29] The P4 Leu residue of the latter occupies the D-S3 subsite as defined by D-Phe-Pro-Arg chloromethyl ketone bound to thrombin[27] thus making it also the L-S4 substrate binding site of the enzyme.

with Rigaku data processing software.[24] The diffraction extends to 2.1 Å resolution (15 999 reflections, 70% completeness, R_{merge} = 8.2%). The structure was solved by isomorphous molecular replacement methods using the hirugen−thrombin structure as a model (1HGT.PDB) and initially refined by rigid body techniques to R = 20.0% in the 9.0−2.8 Å range. The 298-A molecule was included in calculations at this stage from electron density maps. Subsequent restrained least-squares refinement (PROLSQ)[25] gave a final model containing 177 water molecules and a full occupancy 298-A ($\langle B \rangle$ = 31.5 compared to 29.6 Å^2 for thrombin) with R = 0.150 (reflections $I/\sigma > 2.0$ in the 7.0−2.1 Å resolution range). The rms deviations of bond lengths, bond angles, and torsional angles from ideality correspond to 0.018 Å, 3.1° and 24°, respectively. The final model was examined with PROCHECK[26] for orientational inconsistencies of residues and corrected.

The electron density unequivocally displayed the position and orientation of 298-A in the active site of thrombin (Figure 1). Preliminary mass spectrometry and NMR experiments revealed some relative stereochemistry,[5] but no absolute stereochemistry, except for the L-Leu. The absolute configuration of the 298-A has been here determined with respect to the thrombin molecule. The peptide binds to thrombin in a noncovalent way forming an antiparallel β-strand with thrombin resembling the overall binding of D-Phe-Pro-Arg chloromethyl ketone[27] and other similar peptides to thrombin.

The argininol group fits into the S1 specificity pocket and the carboxy-6-hydroxy-octahydroindole (Choi) generally occupies the S2 site (Figure 1). Very unexpectedly, the side chain of L-Leu partially occupies the D enantiomorphic S3 subsite, which is also the L-S4 binding subsite (Figure 2), and Hpla at P4 binds like a P3-L enantiomer at the L-S3 subsite comparable to the aspartate of the Leu-Asp-Pro-Arg sequence of thrombin platelet receptor[28] (Figure 2). The guanidine of the argininol forms a doubly hydrogen bonded ion pair with Asp 189 of thrombin, while its hydroxyl group makes a hydrogen bond with His57NE2 displacing Ser195 OG; the latter then appears to make a transition state interaction with the argininol carbon atom (2.8 Å) (Figure 1). As is usual for proline groups at P2, the prolyl of Choi is flanked by the hydrophobic S2 binding subsite[27] while its six-membered ring interacts with Try60A and Trp60D, causing the 60 insertion loop to move. The Gly216−Gly219 stretch in thrombin displays antiparallel β-strand hydrogen-bonding interactions with the 298-A peptide. Correspondent amide and carbonyl groups of the L-Leu residue interact with Gly216, while the secondary hydroxyl of the 3-(4-hydroxyphenyl)lactic acid (Hpla) residue hydrogen bonds with Gly219N. The new and unusual manner in which the L-Leu and Hpla groups of 298-A bind with thrombin (Figure 2) again reveals that sequence or stereochemistry alone does not always predict binding properties.[29,30] This is in agreement with thrombin being generally tolerant of imprecision[31] and adds additional flexibility to the repertoire of structure-based drug design.

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