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A Noncleavable Retro-Binding Peptide That Spans the Substrate Binding Cleft of Serine Proteases. Atomic Structure of Nazumamide A: Human Thrombin‡

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Abstract: The 2.0 Å resolution crystal structure of the retro-binding natural product nazumamide A (NAZA) complexed with human thrombin is presented. The crystal structure shows that the retro-binding nazumamide A is noncleavable and extends into the prime end of the substrate binding cleft. The data suggest ideas for SAR and combinatorial modification of nazumamide A to create a second generation of nazumamide-like compounds which are potent and specific for human thrombin. This crystal structure indicates that fresh ideas for the generation of novel and specific inhibitors may arise from examining weak binding compounds. Additionally, the crystal structure demonstrates the utility of crystallographic analysis of natural product:protein complexes.

Thrombin is central to the regulation of blood coagulation and has thus been targeted for the treatment of various hemostatic disorders such as myocardial infarction, stroke, and pulmonary embolism.[1] Although a number of potent thrombin inhibitors have been reported,[2] many lack a high selectivity over other trypsin-like enzymes. Hence, in order to design a viable therapeutic agent, the issue of specificity must be addressed. One avenue toward this goal is to prepare a noncleavable inhibitor that spans the site of proteolytic cleavage[3] thereby giving access to additional binding loci for the engineering of specificity. Spanning the catalytic site has been successfully achieved for the hirugens and hirulogs where a noncleavable peptide-bond analog was incorporated between P_1 and P_1'[4] (Figure 1A). However, limited oral bioavaliability has curbed their therapeutic usage even though these inhibitors have been shown to be specific for thrombin.

Another strategy for preparing a noncleavable ligand is suggested by the amino acid sequence (Figure 1B) of the recently isolated (marine sponge *Theonella* sp.) and synthesized natural product nazumamide A (NAZA[5]). NAZA has been shown to inhibit thrombin (IC_{50} of 4.6 μM[6]) but not trypsin at concentrations up to 165 μM.[5a] Serine proteases cleave

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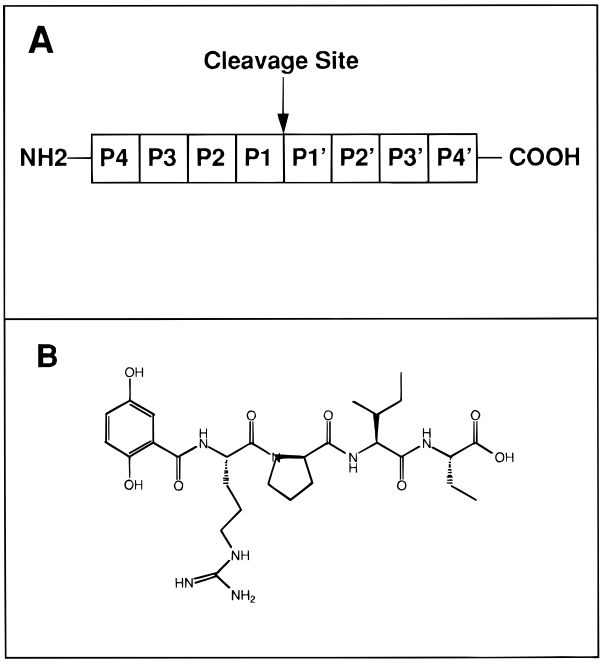

Figure 1. A. Orientation and cleavage site of a typical peptide substrate and B. structural drawing of nazumamide A.

substrates at the amide bond between P_1 and P_1' with, in the case of thrombin, an arginine or lysine being preferred at position P_1 and a proline preferred at position P_2.[7] Hence, one may predict that NAZA COOH-R-Pro-Arg-R′-NH_2 may bind to thrombin in a "retro-" fashion or opposite to typical peptide ligands, i.e., NH_2-R-Pro-Arg-R′-COOH (see Figure 1). This retro-binding mode would give rise to two phenomena: (1) the antiparallel β-sheet hydrogen bonds observed between typical ligands and the backbone rim of thrombin's S_1 pocket[8] would be disrupted and (2) the ligand might be expected to be noncleavable because of the presence of a retro-peptide bond between P_1 and P_1'. Although a decrease in binding energy would coincide with the loss of antiparallel β-sheet hydrogen bonds, the ability to build into the prime side of the binding cleft could allow one to further engineer specificity. To test these ideas as a foundation for the design of more potent and specific serine protease inhibitors in the future, the crystal structure NAZA:thrombin was determined at 2.0 Å resolution.

Results and Discussion

In Figure 2, an F_o-F_c omit electron density map contoured at 2.3σ around NAZA shows that the arginine, proline, and isoleucine side chains as well as two conformations of *N*-2,5-dihydroxybenzoate are well defined in the density, while the α-aminobutyrate group is poorly defined. Figure 2 also shows that NAZA is a retro-binder: the **C**-terminal α-aminobutyrate group is bound in thrombin's S_4 pocket, the isoleucine in S_3, the proline in S_2, the arginine in S_1, and the 2,5-dihydroxybenzoate in S_1'. As expected, the antiparallel β-sheet hydrogen bonds observed with typical peptide ligands are not present in the NAZA:thrombin complex. However, there is a long polar interaction (3.7 Å, not shown) between the carbonyl oxygen of $ProP_2$ and Glu216-N.[9] Aside from this interaction, there are no other hydrogen bonds suggesting parallel β-sheet formation. Thus, stabilization of the NAZA:thrombin complex appears to primarily arise from interactions at the S_x pockets.

At S_1', the electron density suggests two conformations for the *N*-2,5-dihydroxybenzoate (DHB) group. In one conformation (Figure 2), NAZA-DHB is in a weakly-polar edge-to-face interaction with the side chain of Trp60D. Although the distance between the two ring centroids, 5.2 Å, is well within the acceptable range of 3.4−6.5 Å, the relative orientation of the two aromatic rings is not.[10] Ideally, the partial positive edge of one ring should point directly into the partial negative face of the other resulting in an interplanar angle of 90°, 55°, or 20°. In the NAZA:thrombin complex, the interplanar angle is acceptable at 63.2°. However, the edge of Trp60D is not pointing directly into the face of NAZA-DHB and is instead pointing toward the carbonyl carbon of the DHB group. Although the nature of this interaction is difficult to quantitate, the binding energy gained from the Trp60D NAZA-DHB interaction could be a key driving force in the selectivity of NAZA for thrombin over trypsin since the latter enzyme lacks the Trp60D insertion loop. Also in this conformation, DHB-5-OH is accepting a hydrogen bond from Lys60F-Nζ (3.3 Å). However, it is unlikely that this hydrogen bond contributes significantly to NAZA thrombin binding, because it is solvent exposed and the geometry is nonideal. For the second conformation (Figure 2), there is little hydrophobic contact between the DHB ring and thrombin. In this case, a hydrogen bond between DHB-5-OH and Leu41-O (2.9 Å) appears to be the stabilizing force. Hence, each conformation is stabilized differently: hydrophobic, edge-to-face interactions for conformation one and a polar, hydrogen bond interaction for conformation two.

Contiguous density for NAZA at the cleavage site indicates that the retro-peptide bond of this compound remains intact when bound to thrombin. Furthermore, noncontinuous density and a distance of 4.0 Å between the DHB carbonyl and Ser195-OH indicates the lack of covalent bond formation. This is as predicted for retro-binding peptides since the $ArgP_1$ amide nitrogen would be present in place of the carbonyl for typical nonretro substrates.

At S_1, a novel hydrogen bonding pattern was observed for the NAZA:thrombin complex (see Figure 3). Because NAZA is a retro-binder, it is missing one atom between the $ProP_2$ ring and $ArgP_1$-Cα[11] resulting in NAZA-$ArgP_1$'s side chain being shifted out of the S_1 pocket by about 1.7 Å relative to $ArgP_1$ of ligands such as the covalent binding H-(D)Phe-Pro-Arg-CH_2-Cl[8] or the noncovalent binding H-(D)Phe-Pro-Arg.[12] Accordingly, the NAZA-$ArgP_1$ side chain is approximately one methylene too short for its guanidinium group to engage in a direct salt-bridge with Asp189, and although the interaction is solvent-mediated, it is different from that between Asp189 and Ac-(D)Phe-Pro-boro$LysP_1$ (Figure 3B[13]). Even though boroLys-Nζ and NAZA-$ArgP_1$-NH_1 are nearly superimposable, $ArgP_1$-NH_2 displaces the water molecule that connects Ac-(D)Phe-

(6) Under typical assay conditions for this study (0.10 M sodium phosphate buffer, pH 7.5, 0.20 M sodium chloride, 0.5% PEG8000, 25 °C, 0.12 nM thrombin, and 0.20 mM S-2238, H-(D)Phe-Pip-Arg-pNA, or S-2366, pyroGlu-Pro-Arg-pNA) no notable thrombin inhibition was observed. Data were suggestive of a K_i in the mM range which is much higher than the reported IC_{50}. However, the clear and continuous electron density present in the initial difference Fourier maps indicated the presence of a NAZA thrombin complex.

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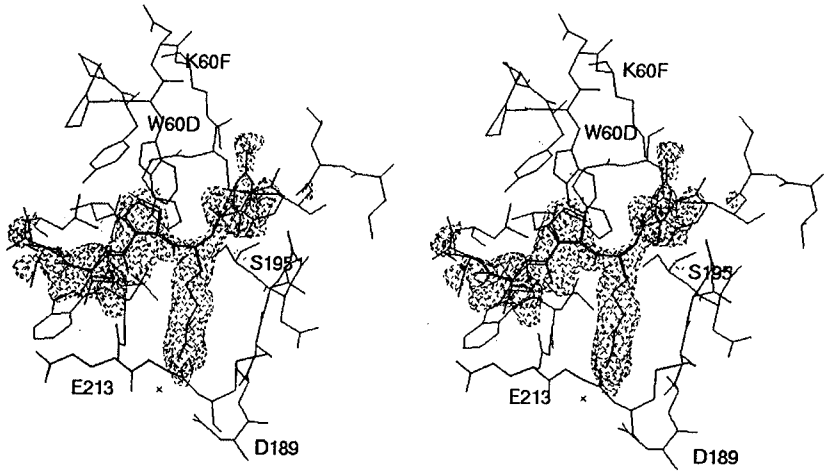

Figure 2. Stereo drawing of nazumamide A: thrombin. A simulated annealing F_o-F_c omit map (2.3σ) calculated upon completion of refinement is shown contoured around both conformations of NAZA.

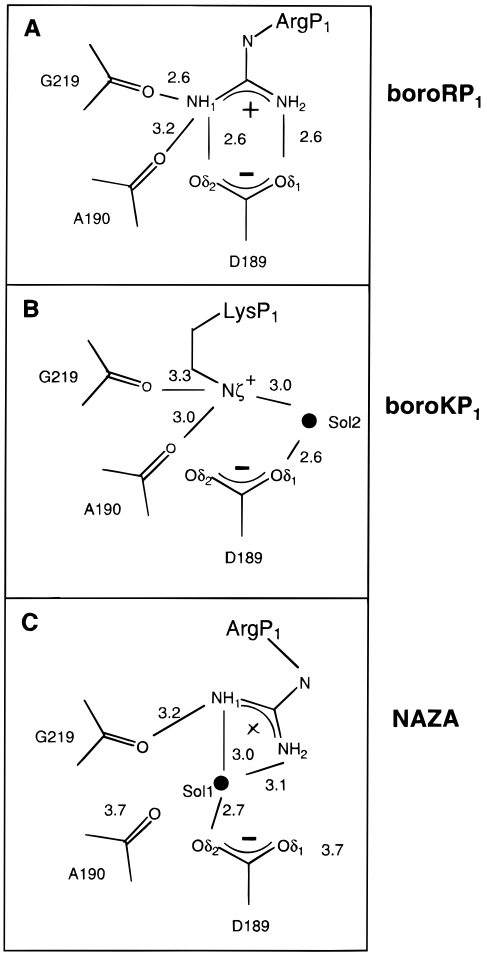

Figure 3. Diagram of the hydrogen bonding observed in the S_1 pocket of thrombin between A. a typically $ArgP_1$ containing peptide (Ac-(D)-Phe-Pro-boroArg),[13] B. a typically $LysP_1$ containing peptide (Ac-(D)-Phe-Pro-boroLys),[13] and C. the arginine of NAZA.

Pro-boro$LysP_1$ with Asp189-Oδ_1 such that a different ordered solvent intervenes between NAZA-$ArgP_1$-NH_1, -NH_2 and Asp189-Oδ_2. Thus, unlike typical $ArgP_1$ peptide inhibitors (Figure 3A) where both nitrogens are hydrogen bonding with Asp189 or unlike nonpeptidic 4-TAPAP,[14] MD-805,[15] and the retro-binding BMS-183507[16] where one guanidinium nitrogen is donating a hydrogen bond to Asp189, NAZA-$ArgP_1$ makes **no direct** hydrogen bond with Asp189. For NAZA, the binding of the DHB group at S_1' may stabilize the unusual binding mode of $ArgP_1$.

The orientation of the NAZA-$IleP_3ProP_2$ is as expected for typical nonretro-binding thrombin ligands. The Pro-P_2 side chain extends into the hydrophobic S_2 site as described for H-(D)Phe-Pro-Arg-CH_2-thrombin,[8] and $IleP_3$ is in contact with the hydrophobic portion of the side chain of Glu213 as observed for the *tert*-butyloxycarbonyl (Boc) group of Boc-(D)Phe-ProArg.[12] Thus, although the anti-parallel β-sheet hydrogen bonds observed for typical peptide substrates have been lost for NAZA, interactions at the S_2 and S_3 specificity sites remain very similar and should contribute to the binding affinity of this compound.

In Figure 2, the α-aminobutyrate (α-$ABUP_4$) group is presented in two possible orientations although the poor quality of the density at this site does not specify or preclude either orientation. The disorder of the density at P_4 can be explained by the lack of a counter-charge for the negatively charged C-terminus. In one orientation (Figure 2), the binding mode for the C-terminus is unfavorable as it is partially buried in the hydrophobic S_4 pocket. However, for the opposite orientation where the α-aminobutyrate ethyl group is oriented deeper in S_4, the negative charge is pointing toward the hydrophobic side chain of Ile 174. Hence, interactions of the carboxyl group in both orientations are unfavorable. This suggests that the α-aminobutyrate group may destabilize the binding of NAZA to thrombin.

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Conclusions

Here, the crystal structure of NAZA:thrombin demonstrates that NAZA binds to thrombin in a novel retro-manner. NAZA is not the first retro-binder for which a crystal structure has been reported. BMS-183507[16] which binds to thrombin with high affinity (K_i = 17.2 nM) was previously shown to orient in a retro-mode by X-ray crystallography. However, unlike NAZA, this compound does not extend into the prime end of the binding cleft. In addition, unlike NAZA, BMS-183507 was shown to form a true parallel β-strand with the thrombin main chain segment Ser214-Gly219. A similar parallel arrangement has also been observed between the N-terminus of hirudin and thrombin.[17] This main chain segment corresponds to that which forms an anti-parallel β-strand with typical peptide ligands such as (D)-Phe-Pro-Arg-CH_2-Cl.[8]

In summary, the primary binding forces driving NAZA:thrombin complex formation appear to be interactions at the S_1', S_1, S_2, and S_3 pockets with little or no contribution from S_4. However, because the binding at S_1', S_1, and S_3 appears nonoptimal and because α-ABU may actually deter binding, this structure suggests many ideas for second generation NAZA-like compounds. At P_3-P_4, replacing the leucine-α-aminobutyrate groups with a blocked, neutrally charged phenyl group should increase the binding potency by maximizing hydrophobic contact at S_4 as first discussed for H-(D)PheProArgCH_2-thrombin[8] as well as by eliminating the uncompensated negative charge of the C-terminus. $ProP_2$ appears to need no modification although at P_1, the use of homoarginine should increase the binding energy by maximizing hydrogen bonding interactions in the S_1 pocket. Finally, because $DHBP_1'$ is not uniquely bound and because the prime side of the binding cavity extends beyond the location of this group, it is an ideal candidate for extended SAR. This would allow a detailed characterization of the binding properties of this cavity while identifying more potent and specific thrombin inhibitors.

Examination of the crystal structure of NAZA-thrombin strongly reinforces the point that fresh ideas for novel inhibitor design may be obtained by structural characterization of natural product protein complexes. Here, we present the complex of a noncovalent inhibitor which extends into the prime side of a serine protease substrate binding cleft by taking advantage of the noncleavable properties of a retro-binder. The NAZA:thrombin investigation also strongly supports completion of weak binding inhibitor complex crystal structures. It is clear that the weak binding properties of NAZA make it a nonviable anticoagulant. However, NAZA may serve as a useful template for the design of more potent and specific thrombin inhibitors as well as inhibitors of other serine proteases via both synthetic and combinatorial methods.

(21) We thank Frank Lewandowski for growing the thrombin crystals and Drs. Richard Alexander and Robert Galemmo for helpful discussions. V.L.N. thanks the DuPont Merck post-doctoral program for financial support.

Experimental Section

Synthesis. NAZA was synthesized in a manner analogous to the route used by Hayashi and co-workers.[5b] However, conventional peptide coupling methods were employed in place of the diethyl phosphorcyanidate method. All intermediates were characterized by ^{1}H NMR, low resolution mass spectrometry, and high resolution mass spectrometry. Optical rotations match those reported by Hayashi and co-workers.[5b]

Crystallographic Studies. Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN) and used without further purification, while the hirudin C-terminal peptide, residues 54−65, was synthesized in house by Rose Wilk of the DuPont Merck Biotechnology Peptide Synthesis Facility. The thrombin−hirudin complex was crystallized in the space group C_2 (a = 70.67 Å, b = 72.9 Å, c = 73.0 Å, β = 100.4°) by the procedure of Skrzypczak−Jankun *et al.*[18] with some modifications. To prepare thrombin−inhibitor complexes, thrombin crystals were soaked in a solution containing 2.0 mg of nazumamide in 250 μL of crystal stabilization buffer (0.58 M sodium phosphate pH 7.2, 33% polyethylene glycol MW 8000, 0.05 mM NaN_3) for 1 week prior mounting crystals in capillaries. Inhibitors were initially solubilized in DMSO. In order to maximize bound inhibitor, a small amount of nazumamide powder was placed in the capillary next to the crystal during data collection.

X-ray diffraction data for the thrombin−nazumamide complex was collected on an R-axis Image Plate system mounted on a Rigaku RU-200 rotating anode generator operating at 100 mA and 50 kV. Twenty min two degree oscillation frames were collected for a 120° coverage at two crystal orientations. The data were then reduced using software supplied by the manufacturer. Crystals were sufficiently stable in the X-ray beam so that only one crystal was needed to collect a complete data set, 95 619 accepted observations $>0.5\sigma$ of 92.5% of 21 475 reflections with an R_{sym} of 8.68% based on F^2 to 2.0Å resolution.

Initial difference Fourier maps were computed using the structure factors and phases calculated from the refined coordinates of the ternary complex between Ac-(D)Phe-Pro-**boro**Arg-OH, human α-thrombin, and the hirudin peptide[13] with the inhibitor removed. Inhibitors were placed in the initial difference electron density maps using the interactive graphics package TURBO,[14] and the coordinates were refined using the program package XPLOR[15] with the parameter sets parhcsdx.pro and tophcsdx.pro. Refinement of the complexes continued with alternate cycles of positional and individual *B*-factor energy minimization at 2.0 Å resolution. The final 2.0 Å structure was well refined as indicated by an *R*-factor of 19.5% with rms bond lengths of 0.017 Å and rms bond angles of 2.9°. Ordered solvent molecules (130 total) were located as positive peaks which were at least 4σ above noise in difference electron density maps during the course of the refinement.

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