Crystallographic Structures of Human α-Thrombin Complexed to Peptide Boronic Acids Lacking a Positive Charge at P1. Evidence of Novel Interactions

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Received April 28, 1997

Human α-thrombin (HαT) catalyzes the cleavage of Arg−XX bonds, and a common feature in the design of specific inhibitors has been the introduction of a positively charged group at P1[1] to emulate the ion pair formed between the guanidino group and the carboxylate side chain at the base of the S1[1] specificity pocket. A good example is the peptide boronic acid Ac-D-Phe-Pro-boroArg (DUP-714).[1,2] In early studies with various aromatic analogues of the arginine side chain, it was observed that these moieties (e.g., benzamidine) exhibited higher affinity for HαT than aliphatic congeners.[3] This suggests that additional hydrophobic interactions are possible within the S1 site. We report here that, in contrast to other trypsin-like serine proteases, peptide boronic acids lacking a positive charge at the P1 site can be designed which are potent inhibitors of HαT.

Upon analysis of the structure−activity relationship of a series of tripeptide boronic acids with uncharged P1 side chains,[4a] the polar aprotic side chain, methoxypropyl, was found to be the most selective for HαT.[4] The crystallographic analysis of the complex (**1**) of HαT with Moc-D-Dpa-Pro-boroMpg,[1] compound **1** (Figure 1) ($K_{i,Thr}$ = 3 nM),[4a] at 1.9 Å resolution, provides for the first time some explanation for the basis of the high-affinity interaction at the S1 site which is not seen with a shorter ethyl side chain, (*Z*)-D-Dpa-Pro-boroEtg[1] ($K_{i,Thr}$ = 2000 nM). Surprisingly, crystallographic analysis at 2.1 Å of the complex (**2**) with (*Z*)-D-Dpa-Pro-boroVal,[1] compound **2** (Figure 1) ($K_{i,Thr}$ = 20 nM), shows a novel interaction in which covalent bonding to the catalytic triad of thrombin is prevented, even though moderate affinity is achieved.

Tripeptide boronates **1** and **2** were synthesised as described,[4a,5] and crystals obtained for **1** and **2** with HαT and *N*-Ac-hirugen, by vapor diffusion using the hanging drop method, with 25%

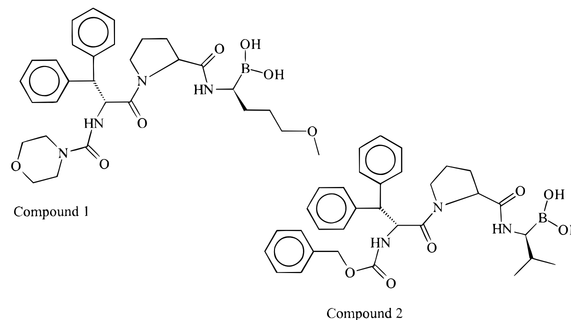

Figure 1. Tripeptide boronate thrombin inhibitors compound **1**, Moc-D-Dpa-Pro-boroMpg[1] ($K_{i,Thr}$ = 3nM),[4a] and compound **2**, (*Z*)-D-Dpa-Pro-boroVal[1] ($K_{i,Thr}$ = 20 nM).[4a]

PEG 8000 (w/v), 0.05 M ammonium phosphate (pH 7.3), and 0.05 M sodium azide. Crystals were prepared for flash cooling by soaking in crystallization buffer with the addition of 25% PEG 400. Data sets were collected to a maximum Bragg spacing of 1.8 and 2.1 Å for compounds **1** and **2** with HαT. Ac-hirugen was subsequently processed with Denzo[6] and Scalepack.[6] Initial molecular replacement solutions were determined using AMORE[7] with the ternary complex of HαT·desaminohirugen·PPACK.[8] Intitial *R*-factors of 0.36 and 0.34 were reduced by rigid body refinement to 0.32 and 0.30 for complexes of compounds **1** and **2**, respectively. Weighted difference maps clearly showed extra density for the inhibitor at the active site of the protein. Inhibitor coordinates were obtained using the program Spartan,[9] the complex was refined using Refmac,[7] and water molecules were added using ARP.[10] Refinement[7] of the complexes converged to a crystallographic *R*-factor of 17.5% (R_{free} = 24.0%, using 5% reflections) and 17.0% (R_{free} = 23.5%) for complexes **1** and **2**, respectively. Root mean square deviations from ideal values of bond lengths and angles were 0.02 Å and 2.4° and 0.019 Å and 2.5° for the complexes **1** and **2**, respectively. Atomic coordinates have been accepted in the Brookhaven Protein Data Bank with accession numbers 1ai8 and 1aix for compounds **1** and **2**, respectively.

Both compounds **1** and **2** form the canonical interactions with human α-thrombin at the S2 and S3 sites, already shown with the PPACK complex.[11] The *pro-R*-phenyl of the P3 Dpa group forms an edge-on aryl−aryl interaction with Trp-215, and the P3 peptide backbone forms an antiparallel interaction with Gly-216 (P3 αNH−Gly-216-CO 2.94, 2.66, and 2.66 Å and P3 αCO−Gly-216-NH 3.30, 2.95, and 3.11 Å for the complexes of compounds **1**, **2**, and PPACK,[11] respectively). The P2 proline is well accommodated in the S2 site in both structures, forming hydrophobic interactions with the Tyr-60 D loop and His-57. The P1 α-amino forms a hydrogen bonding interaction with the carbonyl of Ser-214 (2.99, 3.4, and 2.87 Å for compounds **1**, **2**, and PPACK,[11] respectively). These interactions in the S2 and S3 sites mimic those of the productive enzyme substrate complexes of HαT with fibrinogen.[12]

Complex **1** shows the expected covalent interaction of ca. 1.75 Å identified by continuous electron density between the boron and the Ser-195 Oγ of the HαT catalytic triad (Figure 2), as previously reported for positively charged peptide boronates with HαT[13] and in peptide boronate complexes with other serine proteases. The boronate oxygen O1B is coordinated

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(1) Terminology: designation of the first residue of the ligand as P1 interacting with the S1 position of the protein is from the established protocol of I. Schechter and A. Berger (*Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162). α-Aminoboronic acid or boro(aa) is where the CO_2 of the equivalent amino acid is replaced by BO_2. Abbreviations: Mpg, (3-methoxypropyl)glycine; Etg, ethylglycine or homoalanine.

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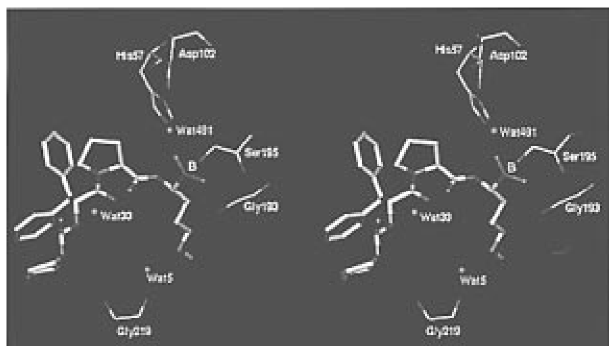

Figure 2. Stereoview of the active site region of ternary complex **1** of HαT·Ac-hirugen·**1**. Compound **1** is shown complete, with selected HαT residues. The location of WAT5 (red sphere) between the P1 ether oxygen and HαT Gly-219-CO is indicated.

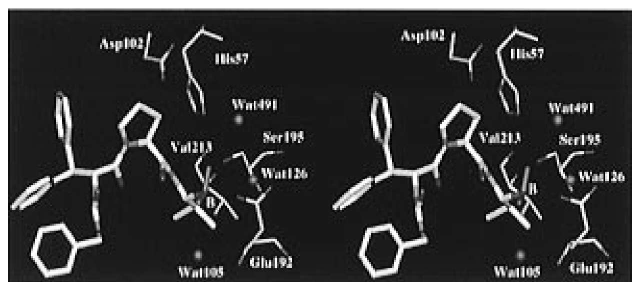

Figure 3. Interactions in the active site region of complex **2** of HαT·-Ac-hirugen·**2**. Compound **2** is shown complete, with selected HαT residues. The proximity of the P1 Val side chain to the position of the HαT Val-213 residue and the interaction of the boronic acid moiety with the carboxylate of HαT Glu-192 are shown.

by Gly-193 NH and Ser-195 NH in the so-called oxyanion pocket (Figure 2) as for the scissile bond carbonyl of substrates and of PPACK (O1B−Gly-193 NH 2.79 Å, O1B−Ser-195-NH 3.11 Å; PPACK P1 αCO−Gly-193-NH 3.22 Å, αCO-Ser-195-NH 3.16 Å for compound **1** and PPACK, respectively). The similarity of these interactions with those proposed for the transition state of the natural proteolytic reaction is considered to explain the nanomolar affinity of this inhibitor, compound **1,** with HαT.

Unlike complex **1**, complex **2** does not show substrate-like interactions at S1. The boron is 3.34 Å from Ser-195 Oγ, and no electron density is observed between the boron and oxygen. Instead the boron oxygen O1B is now displaced from the oxyanion pocket and is hydrogen bonded (2.84 Å) to Ser-195 Oγ (Figure 3), a separation that is significantly less than the van der Waals contact. In the complex **1** between HαT and compound **1**, the B−Ser-195 Oγ interaction places both boron oxygens almost symmetrically about the serine at 2.41 and 2.3 Å for O1A and O1B, respectively. For HαT complexed to compound **2** the displacement allows O1A to interact more strongly with the carboxylate side chain of Glu-192 (O1A−Glu-192-OE1 3.11 Å compared to 4.16 Å for complexes **2** and **1**, respectively). The inhibitor P1 valine-like isopropyl side chain in complex **2** is displaced into close proximity of Val-213 of HαT (Figure 3). Studies of computer-generated random allignment of aliphatic (methane, propane) and aromatic (benzene) hydrophobic vectors,[14] the occurence of a leucine residue at the P1 of the natural HαT inhibitor, heparin cofactor III, and the ability for thrombin to accomodate the bulky tryptophan side chain[15] have shown that the upper portion of the HαT S1 site can form strong hydrophobic interactions away from Asp-189. This may be facilitated by the small apolar residue Ala-190, which in trypsin is a bulkier, more hydrophilic Ser-190.[4c,15] The fact that the binding affinity for compound **2** compared to **1** is within an order of magnitude, despite the loss of a covalent B−O bond for complex **2**, indicates compensation by the hydrophobic and the Glu-192 interactions. The P1 histidine residue of Rhodiin, a potent macrocyclic inhibitor of HαT, (K_i = 0.2 pM),[15] also coordinates to the Glu-192 residue, increasing affinity.

The specificity[4a] of tripeptide boronate inhibitors with uncharged P1 side chains for HαT, compared to that for other trypsin-like serine proteases, appears to be simply a result of the affinity of hydrophobic groups for the specificity pocket of HαT.[3c] Complex **1** reveals however a new set of interactions with the HαT S1 site, which utilizes the amphipathic nature of the S1 site, without an ion pair interaction with Asp-189. In complex **1**, the ether oxygen is functioning as a hydrogen bond acceptor from a water (2.54 Å) which is in turn bridging to Gly-216 CO and Gly-219 CO (Figure 2). This is enabled by full extension of the propyl side chain (P1 Ca−Cd), and explains the augmented affinity of compound **1** compared to a compound with a P1 pentyl side chain[4a] lacking a hydrogen bond acceptor. Significantly, this bridging interaction is also present in the fibrinopeptide A−α-thrombin complex,[12] between the ϵ-NH of the arginine guanidino (3.14 Å, WAT80, and 2.77 Å, WAT228) and Gly-216-CO (3.41 and 3.47 Å, respectively) and Gly-219-CO (2.72 and 2.73 Å, respectively). This water bridge is not formed in the complex of fibrinopeptide A and γ-thrombin (Gly-216 CO is still hydrated, WAT248 is 3.33 Å and the P1 Arg ϵ-NH1 has moved to 3.13 Å from Gly-219-CO). Since γ-thrombin is relatively poor at cleaving fibrinogen, this water bridge may be considered a "conserved" interaction and a primary determinant in the orientation of macromolecular substrates to form catalytically productive complexes with HαT. HαT is known to prefer P1 arginine in substrates, while trypsin prefers P1 lysine which has only a ζ-NH and so cannot bridge to Gly-216, which is the case for BPTI−trypsin complexes.[17]

Mutagenesis studies of trypsin[18] have also concluded that interactions with Gly-216 are fundamental to the catalytic mechanism of serine proteases, such that mimicking the full interactions with the Ser-214-Gly-219 segment may orient the catalytic triad properly in complex **1** to give the covalent interaction with Ser-195, unlike in complex **2**.

Analysis of these complexes elaborates the hypothesis that the S1 site of HαT is amphipathic, with strong hydrophobic interactions in the upper segment, while retaining very hydrophilic interactions, dependent on buried, conserved waters at the "base" of the pocket around Asp-189.

The discovery of this interaction between the P1 residue and the S3 site for human α-thrombin may provide a better understanding of the design of low molecular weight inhibitors of high specificity.

Acknowledgment. We dedicate this paper to the memory of Göran Claeson. This work was supported by the Thrombosis Research trust.

JA9713338

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