Crystal Structure of a Peptidyl Pyridinium Methyl Ketone Inhibitor with Thrombin[†,‡]

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ABSTRACT: The crystal structure of a complex between a bivalent peptidyl pyridinium methyl ketone inhibitor and human α-thrombin has been solved and refined at 2.0 Å to an *R* factor of 0.18. The inhibitor, (D)cyclohexylalanine-Pro-Arg-($CH_2N^+C_5H_4CH_2CO$)-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-cyclohexylalanine-(D)Glu (coded P596), which forms a reversible covalent complex with thrombin, is highly potent with a $K_i = 4.6 \pm 1.0 \times 10^{-14}$ M, lower than that of recombinant hirudin. The N-terminal, active-site-directed portion of the inhibitor is linked to the fibrinogen recognition exosite binding portion by a tetraglycine segment. The strong electron-withdrawing effect provided by the permanent positive charge on the pyridinium nitrogen makes the arginyl carbonyl carbon more susceptible to nucleophilic attack. In the crystal, a covalent P596−thrombin complex is observed. The electron density surrounding the active site portion and the pyridinium of the inhibitor is very well defined, clearly showing the existence of a covalent bond between the Ser^{195} Oγ and the now tetrahedral carbon of the inhibitor. The decreased binding ability of thrombin inhibitors containing N-terminal acetylation is discussed as is the effect of replacing the P_3 (D)phenylalanine with (D)cyclohexylalanine. The electron density surrounding the remainder of the inhibitor is generally well defined, the exceptions being the C-terminal (D)Glu, the highly flexible tetraglycine linker, and some of the solvent-directed side chains. The C-terminal part of the inhibitor corresponds to the exosite-directed inhibitor MDL-28050 [Qiu, X., Yin, M., Padmanabhan, K. P. Krstenansky, J. L., & Tulinsky, A. (1993) *J. Biol. Chem. 268*, 20318−20326], and the two bind to thrombin in a similar way.

Thrombin (EC 3.4.21.5) is a trypsin-like serine protease which plays a central role in hemostasis and thrombosis. The enzyme is multifunctional, not only creating blood clots through the conversion of the soluble circulating fibrinogen into fibrin but also involved in the activation of other proteins. These include factor XIII, protein C, and other coagulation enzymes, such as factors V and VIII (Fenton *et al.*, 1991; Davie *et al.*, 1991). It is involved in the stimulation of platelet secretion and aggregation in blood through a proteolytic mechanism involving the cleavage of its receptor (Vu *et al.*, 1991).

Myocardial infarction is often caused by the blockage of coronary arteries by a thrombus and can be alleviated with the use of tissue plasminogen activator, urokinase, or streptokinase (Coller, 1990; Cairns *et al.*, 1992). Unfortunately, this leads to reocclusion in 10−20% of patients possibly due to the liberation of thrombin which had been trapped in an active form within the clot (Sherry, 1987; Fitzgerald & Fitzgerald, 1989). Gold *et al.* (1986) have indicated that, despite anticoagulation, up to 45% of reperfused arteries undergo rethrombosis. Anticoagulant agents are also routinely used during coronary angioplasty (Topol *et al.*, 1993) and in the prophylaxis of thromboembolic disorders in high risk patients (Wallis, 1989; Hauptmann & Markwardt, 1992). Heparin remains the most commonly used anticoagulant in the clinical setting but shows lower efficacy when compared to other types of thrombin inhibitors. Thrombin bound to a fibrin clot is poorly accessible to the heparin−antithrombin III complex (a major plasma thrombin clearance mechanism) resulting in platelet reactivation and, through the subsequent release of platelet factor 4 and heparinase, a neutralization of heparin (Hogg & Jackson, 1989; Badimon *et al.*, 1991; Mirshahi *et al.*, 1989; Rübsamen & Eschenfelder, 1991; Becker, 1993). Consequently, there is a need for the development of more effective anticoagulants where thrombin is a primary target.

Hirudin, a small 65-residue protein isolated from the leech *Hirudo medicinalis*, is a potent anticoagulant offering significant advantages over other anticoagulants. Hirudin binds to thrombin with a K_i of 2.2×10^{-14} M (Stone & Hofsteenge, 1986) and interacts with two distinct sites on the surface of thrombin: the active site and fibrinogen recognition exosite (FRE).[1] This affords an additional advantage over inhibitors directed solely toward the active site which are less effective in preventing the fibrin−

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[1] Abbreviations: Apt, Arg-($CH_2N^+C_5H_4CH_2CO$); Cha, β-cyclohexylalanine; CT, tetrahedral carbon; FRE, fibrinogen recognition exosite; MD-805, (2*R*,4*R*)-4-methyl-1-[N^{α}-(3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl)-Arg]-2-piperidinecarboxylic acid; MDL-28050, succinyl-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D)Glu; P596, (D)Cha-Pro-Apt-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D)Glu; PPACK, (D)Phe-Pro-Arg-chloromethyl ketone; rms, root-mean-square.

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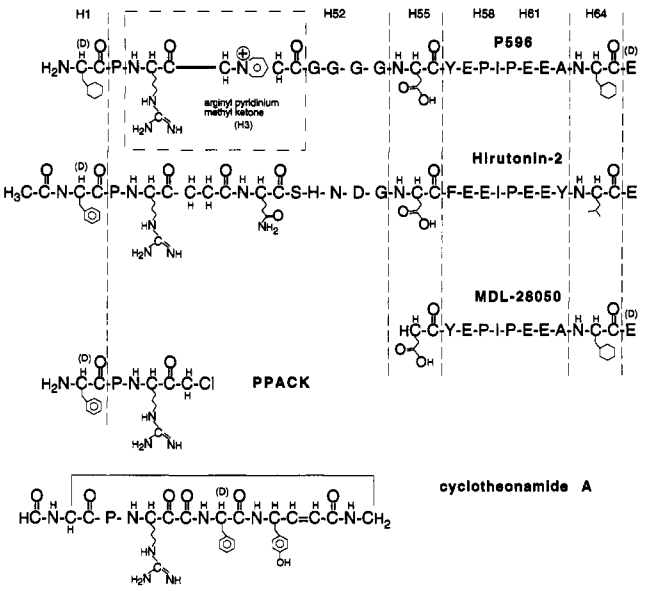

FIGURE 1: Schematic representation of the P596, hirutonin-2, MDL-28050, PPACK, and cyclotheonamide A inhibitors. Amino acids are represented either by a single-letter amino acid code or drawn out; non-amino acids are drawn out. D-Enantiomers are indicated by (D); numbering is based on the hirudin structure.

fibrinogen polymerization due to an unprotected fibrinogen recognition exosite (Kaminski *et al.*, 1991; Fenton, 1992).

Based on hirudin, a class of thrombin inhibitors has been designed in which a small active site inhibitor portion, (D)-Phe-Pro-Arg, is coupled to a FRE inhibitor segment, hirudin,$^{55-65}$ through a short peptide linker (Maraganore *et al.*, 1990; DiMaio *et al.*, 1990). The inhibitor segment and FRE binding part have undergone extensive modifications to enhance potency and proteolytic stability and to reduce the size (Krestenansky *et al.*, 1990; DiMaio *et al.*, 1992, Szewczuk *et al.*, 1992, 1993). A series of modifications designed to completely prevent the proteolytic cleavage of the Arg−X bond were introduced, including the replacement of the P_1 arginine with homoarginine (ArgΨ[CH_2CONH]X), reduced bond analogues (ArgΨ[CH_2NH]X), and ketomethylene pseudopeptide bonds [ArgΨ[$(CH_2)_n$CO]X, $n = 1-3$)] (Kline *et al.*, 1991; DiMaio *et al.*, 1991, 1992), and provided inhibitors with K_i in the range of $10^{-10}-10^{-11}$ M. Significant enhancement of affinity to thrombin was obtained by designing a pseudopeptide bond (ArgΨ[$COCH_2N^+C_5H_4CH_2$-CONH]X) at the P_1-P_1' region (T. Steinmetzer and Y. Konishi, unpublished data). The pyridilacetic acid containing Arg-$CH_2N^+C_5H_4CH_2CO$ group will be referred to as Apt; note the absence of the Ψ indicating a pseudopeptide bond in this alternative nomenclature. The resulting bifunctional inhibitor, (D)Cha-Pro-Apt-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-(D)Glu (P596, see Figure 1), showed a K_i value of $4.6 \pm 1.0 \times 10^{-14}$ M, better than recombinant hirudin $K_i = 2.3 \times 10^{-13}$ M (Braun *et al.*, 1988)] and nearly equal to that of wild-type hirudin. Peptidyl pyridinium methyl ketones were first introduced as substrate analogue inhibitors of proline specific peptidases (Steinmetzer *et al.*, 1993). These types of inhibitors were originally designed to generate a transition state analogue complex when bound to these serine proteases. The incorporation of a permanently positively charged group at the P_1' site and the resulting enhanced electron-withdrawing effect on the P_1 carbonyl group was thought to allow the formation of a reversible hemiketal-like enzyme/inhibitor complex through a nucleophilic attack by the active site serine. The reversible covalent complex formation of the peptidyl pyridinium methyl ketone inhibitors was supported by the slow-binding kinetics (Steinmetzer *et al.*, 1993); however, there was no structural proof available. This proposed mechanism would be similar to that by which trifluoromethyl ketone substrate analogues inhibit chymotrypsin (Brady *et al.*, 1990).

A crystallographic study of the complex between thrombin and P596 was undertaken to provide an explanation of the binding mode of this thrombin inhibitor and, more generally, to illuminate the inhibition mechanism of serine proteases by active-site-directed pyridinium methyl ketones. The FRE inhibitor segment of the P596 inhibitor is nearly identical to MDL-28050 of Krestenansky *et al.* (1990).

MATERIALS AND METHODS

The numbering scheme used for thrombin is based on chymotrypsinogen (Bode *et al.*, 1989). The numbering sequence for the inhibitor is based on the hirudin sequence and prefaced with H. The synthesis, analysis, and kinetic studies of the P596 inhibitor will be described elsewhere. The schematic structure of P596 is given in Figure 1.

Crystallization. Human α-thrombin, obtained from Haematologic Technologies (Essex Junction, Vermont), was used without further purification. The sample, initially at 12.9 mg/mL in 50% glycerol, was dialyzed against 20 mM citrate, pH 5.2, and 1.3 mM K_2SO_4. The inhibitor was added to an inhibitor/thrombin molar ratio of 3:1 yielding a final protein concentration of 6.1 mg/mL. Crystals were grown at 18 °C using the hanging drop method with each drop containing 2 μL of protein solution and 4 μL of reservoir solution. The reservoir solution contained 14.5% (w/v) poly(ethylene glycol) 3350 and 100 mM $KNaPO_4$, pH 6.2. The crystals are monoclinic, space group $C2$, with cell dimensions $a = 70.6$, $b = 72.2$, $c = 72.8$ Å, and $\beta = 100.4°$. There is one molecule of the complex in the asymmetric unit. The crystals are isomorphous to those of the thrombin−hirutonins complexes grown under similar conditions (Zdanov *et al.*, 1993).

X-ray Analysis. Data were collected on a R-axis IIC, mounted on a Rigaku RU300 rotating anode source with Cu target and graphite monochromator. The crystal (0.4 × 0.3 × 0.2 mm) was mounted 90 mm from the detector with the beam collimated by 0.3 mm. The crystal was rotated through 90° in increments of 1.5° and exposure times of 35 min per frame. A total of 43 266 reflections were measured from a single crystal which yielded 19 998 unique reflections with an R_{merge} based on intensities of 8.2%. The completeness of all possible reflections to 2.0 Å is 82.1% with the 2.1−2.0 Å shell 69.0% complete. Data were processed using DENZO (Otwinowski, 1991).

Structure Determination and Refinement. Refinement was carried out using the program X-PLOR v3.1 (Brünger, 1992), fitting to the electron density was performed utilizing the program O (Jones *et al.*, 1991). The α-thrombin from the hirutonin-2 complex used as the initial model gave an R factor of 0.29 for the 8.0−2.0 Å resolution range. Following rigid body refinement, a round of molecular dynamics (MD) refinement with slow cooling from 2000 to 300 K was performed. A round of molecular dynamics involved 60 cycles of minimization, followed by the molecular dynamics refinement, 60 cycles of minimization, 20 cycles of B factor

refinement, and a final 60 cycles of minimization. The crystallographic term, W_A, was determined with the CHECK procedure. The R factor dropped to 0.24, and the electron density clearly showed all of the inhibitor but the linker portion (H51-H55). The shape of the density indicated that there is a covalent bond between the Ser[195] and the inhibitor. However, to avoid bias, Ser[195] was converted to an alanine. Clearly interpretable solvent molecules were added as well. Several further cycles of MD refinement were followed by model refitting to $3F_o - 2F_c$, $F_o - F_c$, and omit maps. At this stage the omit map near residue 195 was carefully inspected, and it convincingly showed the existence of a covalent bond between the Ser[195] and CT atom (carbonyl group) of Arg[H3] with a tetrahedral arrangement of atoms around CT. The alanine at position 195 was converted back to a serine, and the bond between its Oγ and CT was restrained to 1.44 Å with a constant similar to that for covalent C−C bonds. The density for the linker portion of the inhibitor was weaker than for other parts of the inhibitor and, when included with full occupancy, lead to negative density in the difference map. Reducing the occupancy to 0.5 eliminated negative peaks in difference map in this region. The final model included residues 1B to 14K of the A-chain and residues 16−245 from the B-chain of thrombin (with the exception of the autolysis loop, 147 to 149E, which is disordered), all but the N-terminal Glu of the inhibitor, and 186 solvent molecules. The R factor for this model is 0.18 for reflections with $I > 1.5\sigma(I)$ and 0.21 for all reflections, in the 8−2.0 Å shell. Engh and Huber (1991) parameters were used during the refinement, and root-mean-square (rms) deviation for bonds is 0.009 Å and for angles 1.53°. The average temperature factor for the atoms of the complex (excluding solvent) is 23.1 Å^2, while for the water molecules it is 40.4^2.

RESULTS AND DISCUSSION

Thrombin Structure. The structure of thrombin is very similar to that found with previously described α-thrombin−inhibitor complexes (Banner & Hadváry, 1991; Bode *et al.*, 1989, 1992; Grütter *et al.*, 1990; Rydel *et al.*, 1991; Zdanov *et al.*, 1993). As in several other thrombin−inhibitor complexes the autolysis loop is also disordered (Skrzypczak-Jankun *et al.*, 1991; Qiu *et al.*, 1992). When this loop is involved in crystal contacts, the conformation varies from one complex to another (Bode *et al.*, 1991; Rydel *et al.*, 1991). Excluding this loop, the root-mean-square deviation over all Cα atoms between the thrombins of the P596 and hirutonin-2 is 0.24 Å. Only three residues contain atoms deviating more than 2.0 Å; Cys[1] found near the N-terminal, Gly[150] at the end of the autolysis loop, and Lys[186] found in a flexible loop region. Of the remaining residues containing atoms deviating more than 1.0 Å, there appears to be little correlation to interaction with inhibitor. The regions 38−39 and 150−151 do deviate and interact with the inhibitor exosite, but the deviations are not significantly larger than for other loops distant from the inhibitor. The rms deviation, between the thrombin from the P596 complex and that from PPACK (Bode *et al.*, 1992), MDL-28050 (Qiu *et al.*, 1993), and clycloTheonamide A (Maryanoff *et al.*, 1993) are 0.400, 0.402, and 0.313, respectively (see Figure 1 for a schematic representation of the various inhibitors under discussion). The location and degree of these deviations are not that different from those observed between P596 and hirutonin-2 complexes with only a small subset due to differing interactions with the inhibitors (see below). Difference electron density suggested the presence of an oligosaccharide attached to Asn[60G]; however, it could not be identified and was not modeled. The side chain of Arg[75] lies on the 2-fold axis and was therefore changed to an alanine during refinement and fitted to the electron density once refinement was complete. The alternate conformation of this side chain previously described (Zdanov *et al.*, 1993) was not obvious.

Active Site Binding. The electron density associated with the active-site-directed binding portion of the inhibitor [(D)-Cha-Pro-Arg] along with the neighboring thrombin residues is very well defined (Figure 2a). The N-terminal (D)Cha, Pro, and Arg bind to the thrombin S_3, S_2, and S_1 subsites, respectively, with numerous van der Waals, hydrogen bonding, and ionic interactions. The binding is similar to other active site binding inhibitors having the (D)Phe-Pro-Arg motif (Zdanov *et al.*, 1993; Skrzypczak-Jankun *et al.*, 1991; Bode *et al.*, 1989; Qiu *et al.*, 1992; Banner & Hadváry, 1991), yet some differences do occur. The (D)Cha side chain occupies the same site as (D)Phe in hirutonins and PPACK complexes: a hydrophobic cavity defined in the first instance by the side chains Leu[99], Ile[174], and Trp[215] (Figure 3). The cyclohexyl ring adopts a chair conformation, wedged between Leu[99] and Ile[174] and nearly perpendicular to the side chain of Trp[215], which in turn is stacked against Phe[227]. Further hydrophobic interactions are provided by the proline at position 2 of the inhibitor and to a lesser extent by Tyr[60A] and Trp[60D] of thrombin. The cyclohexyl ring is slightly rotated with respect to the plane of the phenylalanine ring in other inhibitors. Due to its saturated character and a nonplanar nature, the cyclohexyl ring hydrogens point directly toward the two neighboring aliphatic side chains resulting in stronger van der Waals interactions than inhibitors with an aromatic ring in this position. The increased affinity to thrombin of inhibitors where (D)Phe is replaced by (D)Cha (Wittig *et al.*, 1992) therefore appears to be the result of van der Waals interactions although the increased hydrophobicity of the cyclohexyl group over the phenyl ring may also be important.

The position of the inhibitor Pro[H2] is defined by hydrophobic interactions provided by its stacking against the indole ring of Trp[60D], by the edge-on contact with Tyr[60A] and His[57], and by Cha[H1]. The aromatic ring of Tyr[60A] is stacked against Pro[60C] and arranged perpendicular to the indole ring of Trp[60D]. The side chain of the inhibitor Arg[H3] is in a fully extended conformation with its guanidyl group forming a well defined salt bridge with the side chain of Asp[189]. Although the possibility exists that the organic synthesis could lead to a racemic mixture of the P1 arginine, the arginine of the bound inhibitor was exclusively in the L-configuration. The NH atoms of the Arg[H3] side chain are further stabilized through hydrogen bonds to the carbonyls of Ala[190] and Gly[219], and through a bridging water to the main chain of Phe[227]. The Nϵ of Arg[H3] hydrogen bonds through a bridging water molecule to the main chain of residues 216−219 and to the Oϵ1 of Glu[192]. This latter residue is bridged to residues H1−H3 of the inhibitor and to Gly[219] of thrombin by several water molecules (Figure 3). The orientation of Glu[192] varies in different thrombin−inhibitor complexes. While in most complexes it is similar to that observed here, in hirutonin-2 (Zdanov *et al.*, 1993)

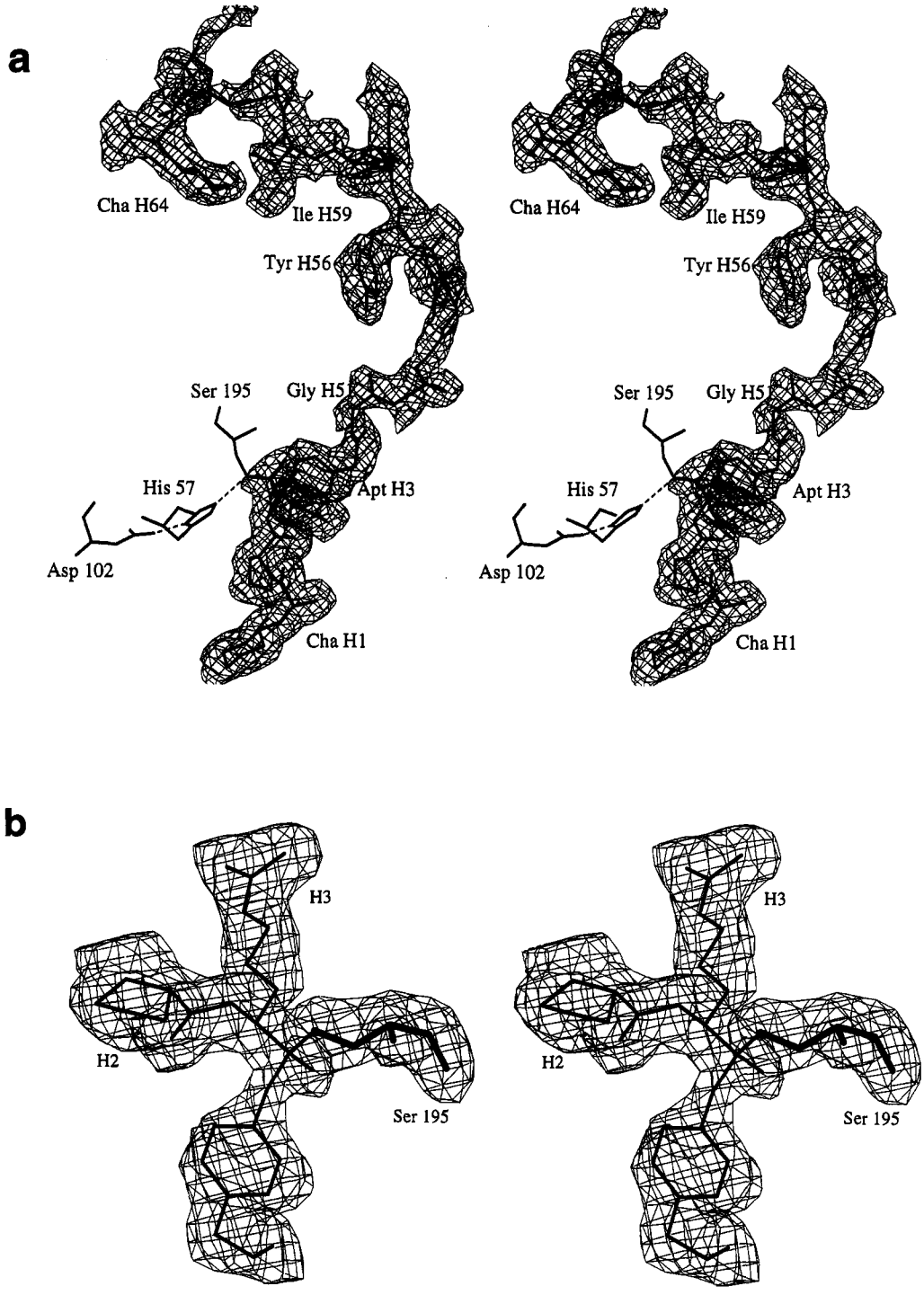

FIGURE 2: Electron density omit maps (minus inhibitor and Ser^{195} of thrombin) contoured at 1.5σ using mapcover radius of 1.0 Å. (a) Entire inhibitor, (b) residues H2 and H3 of the inhibitor and Ser^{195} of thrombin (thick lines) showing the covalent bond between the Ser^{195} Oγ and Apt^{H3} CT.

and MD-805 (Banner & Hadvary, 1991) complexes this side chain is pointing in another direction and is no longer involved in the inhibitor binding.

An important component of the hydrogen bonding network observed with the P596 inhibitor is the free amino group of the (D)Cha in the P_3 position which forms a hydrogen bond to the carbonyl of Gly^{216} and through bridging water molecules to other thrombin side chains. In contrast, the inhibitors hirutonin-2 and hirutonin-6 begin with Ac-(D)Phe-Pro-Arg. Although the $(D)Phe^{H1}$ amide also forms a hydrogen bond with the carbonyl oxygen of Gly^{216}, that is the limit of the involvement of this amide in hydrogen bond formation. The acetyl group displaces the water molecules observed with the P596 inhibitor but the decrease in stability probably has more to do with the observation of Fersht (1987) that amide hydrogen bonds are generally weaker than those formed with free amino groups. The deacetylation of Ac-(D)Phe-Pro-Arg-(CH_2SCH_2CO)-$(Gly)_4$-Asp-Phe-Glu-Glu-Pro-Ile-Glu-Glu-Tyr-Leu-Gln resulted in a ~100-fold improvement in K_i from 1.06 ± 0.096 nM to 9.44 ± 1.21 pM (T. Steinmetzer and Y. Konishi, unpublished data). In addition, the deacetylation of the chromogenic substrate Ac-(D)Phe-Pro-Arg-*p*-nitroanilide resulted in a 26-fold increase in k_{cat}/K_m for the thrombin-catalyzed hydrolysis (Stone *et al.*, 1991).

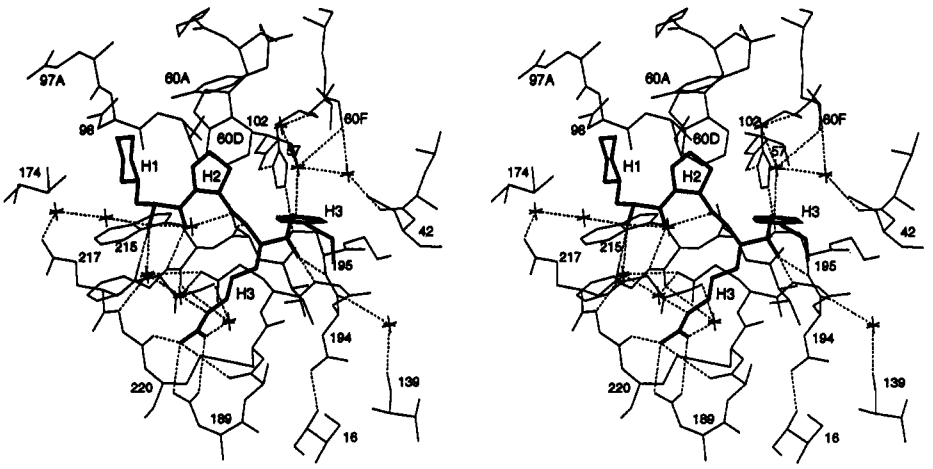

FIGURE 3: Interactions of the active site portion of P596, (D)Cha-Pro-Apt, with thrombin. The inhibitor is shown in thick lines. The Ser^{195} Oγ to Apt^{H3} CT bond is drawn slightly thicker. Position of solvent molecules are marked by asterisks. Dashed lines indicate hydrogen bonds between the inhibitor, protein, and water.

Peptidyl Pyridinium Methyl Ketone. The key to the design of the P596 inhibitor was the incorporation of *N*-methyl-4-pyridiniumacetic acid between the active site binding portion and the linker (Figure 1). This moiety along with Arg^{H3} forms an arginyl pyridinium methyl ketone group which contains a permanent positive charge on the pyridinium nitrogen. The resulting enhanced electron-withdrawing effect on the P_1 carbonyl group allows the formation of a covalent bond through a nucleophilic attack by the active site serine on what was the carbonyl carbon of Arg^{H3}. This carbon in the bound state would be tetrahedral and will be referred to as CT. The Apt abbreviation of the arginyl pyridinium methyl ketone group reflects the transition state form of the bound inhibitor. The omit map clearly shows the contiguous density between the Ser^{195} Oγ and the Apt^{H3} CT (Figure 2b). The good quality of electron density is on par with that observed for the active site binding portion of the inhibitor. The distance between Ser^{195} Oγ and the now tetrahedral carbon of Apt^{H3} is 1.46 Å. The catalytic triad of thrombin is maintained with strong hydrogen bonds being formed between the Nϵ2 of His^{57} and the Ser^{195} Oγ (2.7 Å) and between the Oδ2 of Asp^{102} and Nδ1 of His^{57} (2.6 Å), the latter indicating a possible low-barrier hydrogen bond (Frey *et al.*, 1994). The orientation of Ser^{195} is nearly identical when one compares P596 with hirutonin-2 and the cyclic small molecule inhibitor of thrombin, cyclotheonamide A (Maryanoff *et al.*, 1993). The Oγ atoms are within 0.5 Å of each other with the relative position of the inhibitor determining the distance between the Ser^{195} Oγ and the carbonyl carbon of the inhibitor arginine. In the hirutonin-2 complex, which does not form a covalent bond, these atoms are 2.8 Å apart. Cyclotheonamide A, like P596, forms a covalent bond with Ser^{195} (Maryanoff *et al.*, 1993). The Oγ−C distance was estimated to be 1.8 Å in this case, and the authors suggested a hemiketal-like bond, resembling a postulated transition state geometry (nearly planar arrangement) for the carbonyl carbon. Since at the resolution of 2.3 Å the standard error in atomic position is at least 0.2 Å, the suggested deviation from a tetrahedral geometry has to be taken cautiously. The electron density for the P596−thrombin complex (omit map, Figure 2) indicates a tetrahedral arrangement of this carbon. The Cα position and side chain orientation of the Ser^{195} in P596 complex differ from that of MDL-28050, where the active site is not occupied. In the latter the His^{57} side chain rotates slightly, the end result being a somewhat shorter distance between the Nϵ2 of His^{57} and the Ser^{195} Oγ (2.5 Å) when compared to P596. The position of the Nδ1 of His^{57} and its relation to the Oδ2 of Asp^{102} remain nearly identical between the two complexes. The covalent link between the Ser^{195} Oγ and the carbonyl carbon of the inhibitor's arginine observed with the PPACK complex has the same length (1.42 Å) as in the P596 complex, yet the arrangement differs in that the PPACK is also covalently linked through the methylene to the Nϵ2 of His^{57} (Bode *et al.*, 1992), and the shift of the Ser^{195} is larger than that observed between the P596 and MDL-28050 complexes. The Oδ2 of Asp^{102} forms a further hydrogen bond with the Oγ of Ser^{214} (2.7 Å) while its Oδ1 forms a hydrogen bond to the amides of Ala^{56} (2.9 Å) and His^{57} (2.9 Å). The positive charge on the pyridinium nitrogen is countered to some degree by a water molecule found 2.8 Å above the plane of the pyridinium allowing this generally hydrophobic group to reside in a relatively hydrophobic environment. This water forms a direct hydrogen bond to the Nζ of Lys^{60F} (3.3 Å) and through two other water molecules is hydrogen bonded to the carbonyl oxygen of His^{57} and the carbonyl oxygen of Leu^{41} (Figure 3).

While PPACK is an irreversible inhibitor of thrombin, P596 was shown to be a reversible inhibitor since the P596−thrombin complex slowly dissociated when an excess of a FRE directed inhibitor was added (T. Steinmetzer and Y. Konishi, unpublished data).

Oxyanion Hole Stabilization. The formation of a tetrahedral carbon causes the carbonyl oxygen of Arg^{H3} to become an oxyanion requiring stabilization. These are provided through hydrogen bonds to the amides of Gly^{193} (2.8 Å) and Ser^{195} (2.8 Å). There is also a close contact to the amide group of Asp^{194} (3.3 Å), but the oxygen approaches the amide from a direction nearly perpendicular to the plane of the peptide bond, making a hydrogen bond rather unlikely (Figure 4). The orientation of Asp^{194} is initially defined through the hydrogen bond formation between its side chain and the N-terminal amide of Ile^{16} produced by the cleavage of prothrombin to thrombin. The Asp^{194} side chain also forms a hydrogen bond to the amide of Gly^{142}, while its carbonyl forms hydrogen bonds to the amide of Gly^{196} and, through a water molecule, to the carbonyl of Thr^{139}. This differs from that observed with both hirutonin-2 and PPACK

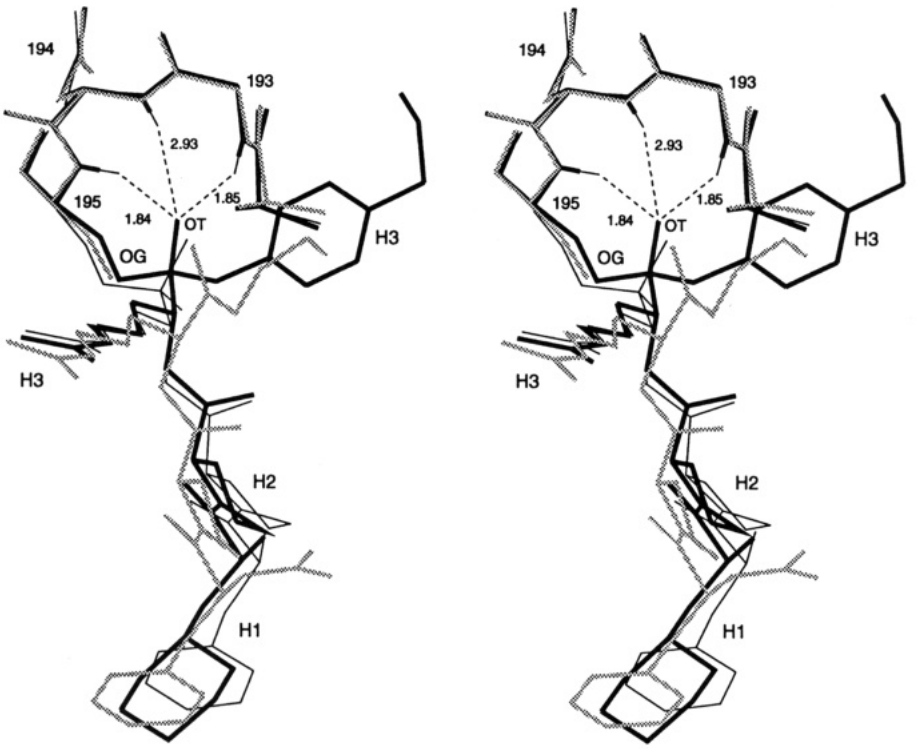

FIGURE 4: Comparison of the interactions of the P596, hirutonin-2, and PPACK inhibitors in the thrombin oxyanion hole. The superposition of the structures is based on the local segment, residues 192−195. The P596 complex is drawn in thick solid lines, the hirutonin-2 complex in thick stippled lines, and the PPACK complex in medium lines. The NH hydrogen atoms of residues 193 and 195 have been included in the figure in their calculated positions. Hydrogen bonds from the oxygen of P596 to these hydrogen atoms are denoted by dashed lines, and their distances are given in angstroms.

in that the oxyanion moves further into the loop defined by residues 192−195 by 0.6 and 1.0 Å, respectively. The hydrogen bonds to the carbonyl oxygen of hirutonin-2 and PPACK are, as a result, much weaker than that seen with P596 (PPACK: Gly^{193}, 3.2 Å; Asp^{194}, 4.1 Å; Ser^{195}, 3.2 Å).

Linker Region. Four glycine residues link Apt^{H3} with the first residue of the fibrinogen recognition exosite binding portion (Asp^{H55}). The density in the "omit" map (no inhibitor included in the calculations) was contiguous throughout the length of the inhibitor, and the linker was modeled into the density. It forms a meander (Figure 2a) with a somewhat unusual conformation and suggest that this, flexible by nature, linker adopts multiple but similar conformations, with the average density as observed in the omit map. To eliminate negative peaks in the this region of the difference map, the occupancy of the linker atoms was set to 0.5. The linker forms few interactions with thrombin and follows the groove formed by residues Gln^{38}−Leu^{41} on one side and Arg^{73}, Asn^{143}, Gln^{151}, and Glu^{192} on the other. While Arg^{H3} and the backbone of Asp^{H55} are in the same position as in other inhibitors, the linker diverges from that of the hirutonins (Zdanov *et al.*, 1993).

Exosite Binding (H55−H65). The fibrinogen recognition site (FRE) binding portion of the inhibitor is identical to the MDL-28050 exosite peptide inhibitor described by Qiu *et al.* (1993), with the exception of Asp^{H55} of P596 being a succinyl group (desaminoaspartic acid) in MDL-28050 in order to remove the N-terminal charge. The electron density corresponding to the inhibitor residues H55−H64 is well defined with the region H55−H60 particularly well ordered. This is especially true for Tyr^{H56} and Ile^{H59} (Figure 2a). The Tyr^{H56} is stabilized mostly through hydrophobic interactions with the thrombin residues Met^{32}, Phe^{34}, Leu^{40}, Arg^{73}, Thr^{74}, and Gln^{151}. The interaction between the hydroxyl group and the guanidyl group of Arg^{73} (3.5 Å) is similar to that observed by Qiu *et al.* (1993). The Ile^{H59} is also buried in a rather hydrophobic environment formed by Phe^{34}, Lue^{65}, Tyr^{76}, and Ile^{82} but sits next to a salt bridge formed by Arg^{67} and Glu^{80}. The hydrophobic environment is further enhanced through the inhibitor itself with Pro^{H60} and cyclohexylalanine Cha^{H64}. The latter interaction likely influences the rotamer of Ile^{H59}, which is identical with P596 and MDL-28050 but differs from that in the two hirutonins. The Cha^{H64} is also stabilized by interactions with Ile^{82}, Met^{84}, and Leu^{65}. The interactions of thrombin with the FRE binding portion of the inhibitor appear to be determined mainly through hydrophobic interactions. As noted by Qiu *et al.* (1993), the negatively charged side chains of Glu^{H57}, Glu^{H61}, and Glu^{H62} do not appear to interact with thrombin although the grouping of side chains around the FRE suggests they may be involved in initial recognition. The side chains of Glu^{H61}, and Glu^{H62} remain disordered. Several hydrogen bonds are formed from the main chain of Glu^{H57} to thrombin. They include a direct bond between the amide of Glu^{H57} and carbonyl of Thr^{74} (2.9 Å) and indirect bonds through a bridging water molecule to the NHs of Arg^{67} and again the carbonyl of Thr^{74}. There is little contact between residues H61−H63 of the inhibitor with thrombin, yet these residues provide a 3_{10} helical turn (Pro^{H60} O−Ala^{H63} N, 2.9 Å) bringing Cha^{H64} into hydrophobic contact with thrombin (see Figure 5). Although the conformation of this helical turn is the same as in hirugen-like inhibitors, its position relative to thrombin differs due to a rotation preceding the 3_{10} helical turn (Qiu *et al.*, 1993). This shift of the 3_{10} helical turn brings the Cha^{H64} side chain to the same hydrophobic site on thrombin as Tyr^{H63} residue in hirugen complexes, although they approach the site from

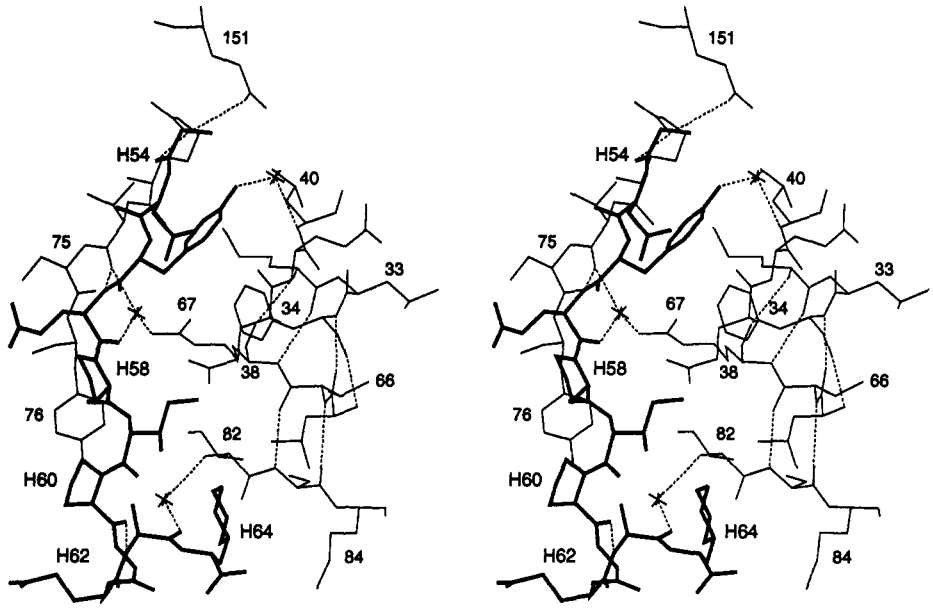

FIGURE 5: Interactions of the exosite binding portion of the inhibitor with thrombin. Inhibitor is shown in thick lines and thrombin in thin lines, asterisks mark positions of water molecules, and hydrogen bonds are drawn as dashed lines.

opposite directions. Interestingly, the position taken by the sulfate group attached to Tyr^{H63} in natural hirudin is occupied in the present complex by a water molecule. In our model the side chain of Asp^{H55} points in the opposite direction to that observed in other inhibitors and does not form a salt bridge to Arg^{73}. Instead, a carbonyl group of the preceding Gly points toward this arginine.

In conclusion, the crystal structure revealed that the high inhibition of thrombin activity ($K_i = 4.6 \pm 1.0 \times 10^{-14}$ M) by P596 results from an enhancement of binding through the formation of a reversible covalent bond between the thrombin active site Ser^{195} Oγ and the carbonyl carbon of the inhibitor arginine, made possible by the incorporation of *N*-methyl-4-pyridiniumacetic acid between the active site portion, (D)ChaProArg, and the tetraglycine linker of the inhibitor. The strong electron-withdrawing nature of the *N*-methyl-4-pyridiniumacetyl group, provided by the permanent positive charge found at this group's nitrogen, made the arginyl carbonyl carbon extremely susceptible to nucleophilic attack. The mechanism is probably the same for the proline specific peptidases (Steinmetzer *et al.*, 1993) for which the peptidyl pyridinium methyl ketone inhibitors were first designed and, by extension, show great potential for other serine proteases.

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