The Role of the Insertion Loop around Tryptophan 148 in the Activity of Thrombin[†,‡]

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ABSTRACT: Thrombin has trypsin-like specificity for Arg-Xaa and Lys-Xaa peptide bonds; however, it is much more specific than trypsin, cleaving far fewer peptide bonds in macromolecular substrates. To probe the nature of the specificity of thrombin, a mutant has been constructed in which the Trp^{148} loop of thrombin has been replaced with the same loop of bovine trypsin. This mutant was expressed in *Escherichia coli* as prethrombin-$2^{(148)}$ using a T7 expression system previously described for wild-type prethrombin-2 [DiBella *et al.* (1995) *J. Biol. Chem. 270*, 163−169]. After refolding and purification, prethrombin-$2^{(148)}$ was activated to thrombin$^{(148)}$ with *Echis carinatus* snake venom. The k_{cat}/K_m for the release of fibrinopeptide A from fibrinogen was 4.5 ± 0.5 μM^{-1} s^{-1} for thrombin$^{(148)}$, which was ∼20% of that of recombinant thrombin (25 ± 2.0 μM^{-1} s^{-1}). Thrombin$^{(148)}$ was inhibited less well by hirudin with a K_i of 500 pM compared to a value of 12 pM determined for recombinant thrombin. The mutant thrombin was also compared to trypsin and wild-type recombinant thrombin for the ability to cleave small peptide substrates. The Michaelis constants (K_m) were found to be between 5- and 10-fold higher for thrombin$^{(148)}$ relative to wild-type recombinant thrombin, although the catalytic constants (k_{cat}) for thrombin$^{(148)}$ and recombinant thrombin remained relatively unchanged for all three substrates. Thrombin$^{(148)}$ had a specificity constant (k_{cat}/K_m) 2-fold higher for the hydrolysis of H-D-phenyalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline (a thrombin substrate) than that of trypsin. For *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroaniline (a trypsin substrate) and *N*-carbobenzoxyglycylprolyl-L-arginine-*p*-nitroaniline (a substrate for both enzymes), the specificity constants for trypsin were 1000- and 16-fold higher, respectively. Although replacement of the Trp^{148} loop does not yield an enzyme with more trypsin-like specificity, the Trp^{148} loop is important in the substrate binding and specificity of thrombin (on the basis of K_m and K_i).

Thrombin (EC 3.4.21.5) is a trypsin-like serine proteinase that plays a major role in blood coagulation (Mann *et al.*, 1988). The thrombin−fibrinogen interaction to form the fibrin clot proceeds in three reversible steps (Scheraga & Laskowski, 1957), with thrombin being involved in only the first one. The specific removal of fibrinopeptides A and B by thrombin exposes complementary polymerization sites near the N-termini of the Aα and Bβ chains of fibrinogen (Laudano & Doolittle, 1980). The fibrin monomer then polymerizes spontaneously, resulting in the formation of an insoluble fibrin clot (Scheraga, 1983, 1986). Thrombin amplifies its own generation by activating the clotting factors V and VIII (Nesheim & Mann, 1979; Hoyer & Trabold, 1981). In addition, thrombin activates the fibrin cross-linking agent factor XIII (Lorand & Konishi, 1964). In the presence of thrombomodulin, thrombin triggers the major anticoagulant pathway by activating protein C (Esmon *et al.*, 1982).

Bovine thrombin is a glycoprotein consisting of two polypeptides, a 259-residue B chain and a smaller 49-residue A chain, connected by a disulfide bond between Cys^1 of the A chain and Cys^{122} of the B chain.[1] The B chain contains all the residues required for catalysis (Hageman *et al.*, 1975; Bode *et al.*, 1989) and is homologous to the catalytic domains of other trypsin-like serine proteases including trypsin, chymotrypsin, and elastase (Jackson & Nemerson, 1980). Using X-ray crystallographic data, the α-carbon structure of human thrombin was superimposed on the structure of bovine trypsin (Bode *et al.*, 1992). On the basis of this superposition, the B chain of thrombin has 195 α-carbon atoms which participate in structurally equivalent residues in the bovine trypsin structure. The rms deviation between the α-carbon backbone of the B chain of thrombin and the structure of trypsin is 0.8 Å. The sequence homology between the enzymes is 36% for thrombin and trypsin.

Although thrombin and trypsin share a high degree of homology, thrombin is much more specific in terms of hydrolysis sites, cleaving far fewer peptide bonds in macromolecular substrates. For example, thrombin cleaves fibrinogen at only four Arg−Gly bonds, whereas trypsin can cleave fibrinogen at roughly 376 Arg−Xaa and Lys−Xaa bonds.

Since the active-site cores of these two enzymes are structurally similar, the differences in specificity might be attributed to the presence of insertion loops on the surfaces

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[1] The chymotrypsinogen numbering of thrombin based on the structural equivalence of thrombin and chymotrypsinogen was employed (Bode *et al.*, 1989). Insertions with respect to chymotrypsinogen were denoted with letters, e.g., Thr^{149A}, Ser^{149B}, Val^{149C}, etc.

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of the two proteins (Bode *et al*., 1989). A feature unique to the structure of thrombin compared to the other serine proteases is its narrow canyon-like active-site cleft shaped mainly by two of these insertion loops, one involving Trp^{148} (residues 145−150) and a second involving Trp^{60D} (residues 50−63).

The Trp^{148} loop is five amino acid residues longer in thrombin than in trypsin and has been proposed to restrict access to the active-site canyon of thrombin, as well as play a role in the overall stability of thrombin (Bode *et al.*, 1992). Evidence for the proposed importance of the Trp^{148} loop comes from X-ray crystallography, in which the loop adopts a different conformation depending on whether thrombin has been complexed with hirudin (Rydel *et al.*, 1990) or PPACK[2] (Bode *et al.*, 1989). Optimal superposition of the hirudin−thrombin structure with that of PPACK−thrombin yields an overall rms deviation of 0.46 Å for α-carbons. The only major difference occurs at the Trp^{148} insertion loop which has an α-carbon rms deviation of 6.3 Å between the two structures.

Two proteolyzed forms of human thrombin result solely from cleavage in the Trp^{148} loop, also termed the autolysis loop; they are (1) ζ-thrombin which results from chymotrypsin or cathespin G cleavage at Trp^{148}-Thr^{149} and (2) ϵ-thrombin, generated by elastase action on Ala^{149A}-Asn^{149B}. A third proteolyzed form, γ_T-thrombin, results from tryptic or autocatalytic cleavage at Lys^{149E}, Arg^{67}, and Arg^{77A}. Virtually all clotting activity is lost upon production of γ_T-thrombin (Hofsteenge *et al*., 1988), while ϵ-thrombin and ζ-thrombin retain ∼85% fibrinogen clotting activity (Stone *et al.*, 1987; Brezniak *et al.*, 1990). In addition, a mutation in the autolysis loop of Glu^{146} to Ala was found to prolong the bleeding time in a patient (Degen *et al.*, 1995).

There are a number of differences in the amino acid sequences of the Trp^{148} loop between human and bovine thrombin. For example, Ala^{149A} and Lys^{149E} are Thr and Glu, respectively, in bovine thrombin. Therefore, different proteolytic forms of bovine thrombin compared to human thrombin are expected. There are two proteolyzed forms of bovine thrombin which have been identified and characterized. One involves cleavage at Thr^{149A} in the Trp^{148} loop producing β_2-thrombin. β-Thrombin, the second proteolyzed form, arises from cleavage at Arg^{77A} and Thr^{149A}. β_2-Thrombin was shown to retain significantly more clotting activity than β-thrombin (Edwards *et al.*, 1986).

In the literature, there have been reports of site-directed mutations being made to the Trp^{148} loop of human thrombin. A mutant of thrombin in which Trp^{148} was replaced by glycine was shown to have slightly modified catalytic activity toward tripeptidyl substrates (Bouton *et al.*, 1995). It was concluded that the thrombin loop 145−150 is indirectly involved in the catalytic function of thrombin, most probably by limiting the access of substrates to the catalytic site. Another mutant of human thrombin in which Glu^{146}, Thr^{147}, and Trp^{148} were deleted (Le Bonniec *et al.*, 1992) was found to have remarkably different catalytic activity toward tripeptidyl substrates. This mutant, des-ETW thrombin was more resistant to inhibition by antithrombin III and hirudin than wild-type thrombin. But, des-ETW thrombin was less resistant to inhibition by bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor. The specific clotting activity of des-ETW thrombin was found to be ∼5% that of thrombin. These studies indicate an involvement of the Trp^{148} loop in the specificity of thrombin.

	143	152
Bovine trypsin	**NT KSSGT----------**	**S YP**
Bovine thrombin	**NR RETWTTSVAE-----**	**V QP**
Thrombin$^{(148)}$	**NR KSSGT----------**	**S QP**

FIGURE 1: Mutation of loop around Trp^{148}. The Trp^{148} loop of bovine thrombin was replaced by the corresponding sequence of bovine trypsin. The alignments of the sequences of bovine trypsin, thrombin, and thrombin$^{(148)}$ are shown. Chymotrypsinogen numbering is used. The dashes do not indicate gaps in the sequence, but simply connect neighboring residues.

In this paper, we describe the construction of a mutant of bovine thrombin in which the Trp^{148} loop of thrombin was replaced by the equivalent loop in trypsin in order to understand the structural basis for the unique specificity of thrombin relative to trypsin (Figure 1). This mutation should provide insight into whether trypsin-like activity can be bestowed on thrombin by the replacement of the Trp^{148} loop. The mutant protein was overproduced in *Escherichia coli* using the same T7 expression system as was reported previously for wild-type prethrombin-2 (DiBella *et al.*, 1995). After refolding and purification, prethrombin-2$^{(148)}$ was activated to thrombin using *Echis carinatus* snake venom. Thrombin$^{(148)}$ was compared to recombinant thrombin in terms of clotting of fibrinogen, release of FpA from fibrinogen, and inhibition by hirudin. To determine the effect of the replacement of the Trp^{148} loop on the trypsin-like activity of thrombin, the kinetics of cleavage of small thrombin and trypsin chromogenic substrates by thrombin$^{(148)}$ have been compared to cleavage by both wild-type recombinant thrombin and trypsin.

MATERIALS AND METHODS

Materials. S2238 was a generous gift from T. Fanella (Kabi Pharmacia), S2222 was purchased from Kabi Pharmacia, and ultrapure guanidine hydrochloride was obtained from ICN Biomedical, Inc. *E. carinatus* snake venom, CbzGPRpNA, and bovine fibrinogen (96% clottable) were purchased from Sigma.

Plasmids and Bacterial Strains. The plasmid pThr-1 encodes for the bovine prethrombin-2 gene (DiBella *et al.*, 1995). M13mp18 was the vector for mutagenesis. Host *E. coli* strains for cloning and mutagenesis were JM101 and Sure (Stratagene). The *E. coli* strain for expression was HMS174(DE3)-pLys(S) (Novagen).

Plasmid Constructs. The *Xba*I/*Hind*III fragment, containing the prethrombin-2 gene, from pThr-1 was cloned into the *Xba*I/*Hind*III-digested M13mp18 producing mThr-1, which was used as the single-stranded template for site-directed mutagenesis. Site-directed mutagenesis was carried out with the T7-Gen In Vitro Mutagenesis Kit (Amersham).

[2] Abbreviations: BSA, bovine serum albumin; CbzGPRpNA, carbobenzoxyglycyl-L-prolyl-L-arginine-*p*-nitroaniline; DMF, *N*,*N*-dimethylformamide; ECEPP, empirical conformational energy program for peptides; FpA, fibrinopeptide A; FpB, fibrinopeptide B; Gdn·HCl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; MALDTOF, matrix-assisted laser desorption time of flight; MUGB, 4-methylumbelliferyl-*p*-guanidinonitrobenzoate; MU, 4-methylumbelliferone; IPTG, isopropyl thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol) 8000; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; S2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroaniline; S2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline.

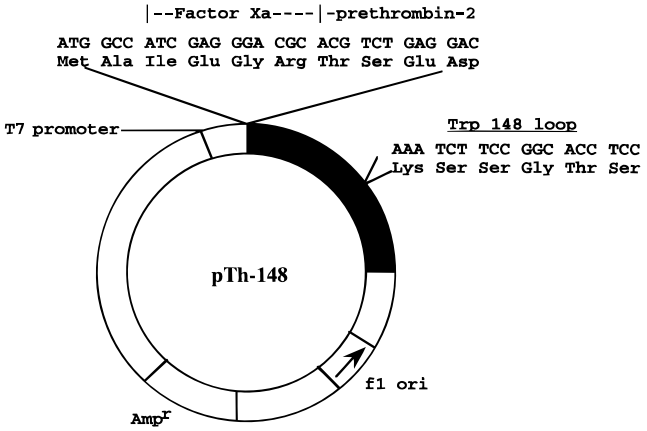

FIGURE 2: Mutagenesis to replace the Trp^{148} loop. Mutagenesis was carried out to change the Trp^{148} loop (autolysis loop) of thrombin to the sequence found in trypsin. pTh-148 encodes for prethrombin-2$^{(148)}$ with a six-residue N-terminal tail of MAIEGR.

The Trp^{148} loop was mutated using the oligonucleotide 5′-CTGGAGGACGCTGGGCTGGGAGGTGCCGGAA-GATTTCCGGTTGCCCCAGCCCGT-3′ after which the *Xba*I/*Hind*III fragment was cloned back into *Xba*I/*Hind*III-digested pThr-1, creating pTh-148 (Figure 2). All mutations and cloning steps were confirmed by dideoxy DNA sequencing.

Expression and Purification of Recombinant Prethrombin-2 and Prethrombin-2$^{(148)}$. Recombinant prethrombin-2$^{(148)}$ was expressed, purified, and refolded essentially as reported previously (DiBella *et al.*, 1995). Briefly, 1 L of 2 × YT (4% tryptone, 1% yeast extract, 10 mM NaCl, and 1.4 mM KCl) containing 200 μg/mL ampicillin and 34 μg/mL chloramphenicol was inoculated with an overnight culture of HMS174(DE3)-pLys(S) transformed with pThr-1 or pTh-148. When the OD_{600} reached 0.6, the cells were induced by the addition of IPTG to 0.4 mM. After an additional 4−12 h, the cell pellets were collected and lysed by sonication. The inclusion body pellets were washed with a solution of Triton X-100 and EDTA, and then the cysteines were sulfonated reversibly (Thannhauser *et al.*, 1984).

Folding and Purification of Recombinant Prethrombin-2 and Prethrombin-2$^{(148)}$. After purification by reverse-phase HPLC, the sulfonated protein was refolded in 4 M Gdn·HCl and 0.1 M sodium phosphate buffer, pH 7.4, containing 2 mM EDTA, 0.1% PEG, 0.1 mM GSSG, and 0.2 mM GSH at room temperature. Folding of wild-type and mutant prethrombin-2 was carried out at protein concentrations from 25 to 100 μg/mL. Yields of active thrombin were the same at all protein concentrations employed. After 16 h at room temperature, the folding solution was dialyzed against 50 mM sodium phosphate buffer, pH 7.4, containing 0.1% PEG and 2 mM EDTA. The refolded protein was purified by heparin affinity chromatography employing a linear NaCl gradient from 0 to 1.0 M in 50 mM sodium phosphate buffer, pH 7.4, containing 0.1% PEG.

Activation to Thrombin and Thrombin$^{(148)}$. The purified, refolded recombinant prethrombin-2 and prethrombin-2$^{(148)}$ (100 μg) were activated to thrombin using *E. carinatus* snake venom (20 μg) at 37 °C for 3 h. The resulting recombinant thrombin and thrombin$^{(148)}$ were purified by heparin affinity chromatography employing a linear NaCl gradient from 0 to 1.0 M in 50 mM sodium phosphate buffer, pH 6.5, containing 0.1% PEG.

Active-Site Titration. The concentrations of thrombin, trypsin, and thrombin$^{(148)}$ were determined by active-site titration with MUGB (Jameson *et al.*, 1973) employing a Perkin-Elmer MPF-44B fluorescence spectrophotometer. The excitation wavelength was set at 323 nm with a slit width of 2 nm, and the emission wavelength was set at 446 nm with a slit width of 6 nm. Trypsin-like serine proteases cleave MUGB releasing the fluorescent molecule MU. The amount of MU released and therefore the amount of active protein present are determined by measuring relative fluorescence and correlating the values to a standard curve of known MU concentrations. The standard curve was determined by measuring the fluorescence of dilutions of a stock solution of MU (20 mM in DMF). The MU stock was first diluted to 0.2 μM in 25 mM sodium phosphate, pH 6.0. At least six dilutions of MU were used in the construction of a standard curve (absolute fluorescence vs MU concentration), and a new standard curve was determined each day. Thrombin, trypsin, or thrombin$^{(148)}$ (15−100 pmol in less than 100 μL) was incubated with 25 μL of MUGB (200 μM in 25 mM sodium phosphate, pH 6.0, diluted from a 20 mM stock in DMF) for 30 min at room temperature in the dark. Fluorescence was measured after dilution to a final volume of 500 μL in 25 mM sodium phosphate, pH 6.0. Since MUGB does hydrolyze nonenzymatically to MU with time, a blank sample of MUGB is used as a reference.

Mass Spectroscopy and N-Terminal Sequencing. Samples for mass spectral analysis and N-terminal sequencing were desalted by reverse-phase HPLC. Data were collected at the Cornell Biotechnology facility using a Finnegan Lasermat MALDTOF mass spectrometer.

Kinetic Analysis of Thrombin, Trypsin, and Thrombin$^{(148)}$. To compare the catalytic efficiencies of thrombin$^{(148)}$, thrombin, and trypsin, kinetic constants were determined for the cleavage of the *p*-nitroaniline peptide substrates S2238, S2222, and CbzGPRpNA. Stock solutions of the chromogenic substrates were prepared in assay buffer (50 mM Tris·HCl, 0.15 M NaCl, 0.1% PEG, pH 7.4), whereas stock solutions of thrombin, trypsin, and thrombin$^{(148)}$ were prepared in the same assay buffer containing 1.0 mg/mL BSA. The hydrolysis of the substrates at 37 °C was monitored by determining the rate of release of *p*-nitroaniline at 405 nm on a modified Cary Model 14 spectrophotometer (Denton *et al.*, 1982).

The amount of product formed was calculated by using an extinction coefficient of 9920 M^{-1} cm^{-1} for *p*-nitroaniline at 405 nm (Lottenberg & Jackson, 1983), and the concentration of peptide substrate was determined by using an extinction coefficient of 8270 M^{-1} cm^{-1} for the three substrates at 342 nm (Lottenberg & Jackson, 1983). Values and standard deviations for K_m and k_{cat} were calculated from triplicate assays by least-squares fit of the data to eq 1 using the program LINFIT (Bevington, 1969).

$$[S]/v = K_m/V_s + [S]/V_s \quad (1)$$

where [S] is the peptide substrate concentration, v is the rate of *p*-nitroaniline release, K_m is the Michaelis constant, and V_s is the maximum velocity.

S2238 Assay. In a typical S2238 assay, thrombin was added to a final concentration of 60 pM, trypsin to 450 pM, and thrombin$^{(148)}$ to 200 pM . Six concentrations of S2238

were used in a range of 1−30 μM for thrombin and 5−300 μM for trypsin and thrombin$^{(148)}$.

S2222 Assay. For the hydrolysis of S2222, thrombin was present at a final concentration of 5 nM, trypsin at 450 pM, and thrombin$^{(148)}$ at 15 nM. The concentration of S2222 varied from 20 to 500 μM for thrombin, 5 to 500 μM for trypsin, and 100 μM to 1 mM for thrombin$^{(148)}$.

CbzGPRpNA Assay. Thrombin and trypsin were present at a concentration of 150 pM and thrombin$^{(148)}$ at 300 pM. The concentration of CbzGPRpNA varied from 10 to 500 μM for thrombin and trypsin and from 100 μM to 1 mM for thrombin$^{(148)}$.

Release of FpA from Fibrinogen and Clotting Times. Thrombin$^{(148)}$ and wild-type recombinant thrombin were compared in terms of their ability to clot fibrinogen and release FpA from fibrinogen. The specificity constant k_{cat}/K_m for the release of FpA from fibrinogen by thrombin and thrombin$^{(148)}$ was determined as described previously (DiBella *et al.*, 1995), with thrombin$^{(148)}$ at a concentration of 12 ng/mL and wild-type thrombin at 3 ng/mL. For determination of a clotting time, 5−100 μL of thrombin$^{(148)}$ or thrombin (0.6 μM) was added to 1 mL of fibrinogen (2 mg/mL in 50 mM Tris, 0.15 M NaCl, 0.1% PEG), and the time necessary for clot formation at room temperature was determined by visual observation. Assays were carried out in triplicate using varying amounts of enzyme. Clotting times are reported in terms of NIH units per mg of protein. One NIH unit of thrombin is defined as the amount that will clot 1.0 mL of a standard fibrinogen solution (~13 μM) in 15 s (Seegers & Smith, 1942).

Inhibition of S2238 Hydrolysis by Hirudin. The inhibition of thrombin by hirudin was followed by S2238 hydrolysis. Thrombin$^{(148)}$ or thrombin (200 pm) in 50 mM Tris buffer, pH 7.8, containing 100 mM NaCl, 0.1% PEG, and 40 mg/mL BSA was incubated with hirudin at concentrations from 10 pM to 3 nM. After 15 min at 37 °C, S2238 was added to 9.2 μM and the release of *p*-nitroaniline followed at 405 nM. The assay with each hirudin concentration was carried out in triplicate.

The K_i for hirudin inhibition of recombinant thrombin and thrombin$^{(148)}$ was determined by nonlinear least-squares analysis of v_s as a function of [I] using eq 2 for a tight-binding inhibitor (Morrison, 1969; Hofsteenge & Stone, 1986).

$$v_S = (v_0/2[E])\{[(K'_i[I] - [E])^2 + 4K'_i[E]]^{1/2} - (K'_i + [I] - [E])\} \quad (2)$$

where

$$K'_i = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (3)$$

and v_s and v_0 are the rates of release of *p*-nitroaniline in the presence and absence of hirudin, respectively. [E] is the concentration of thrombin, [S] is the S2238 concentration, and [I] is the concentration of hirudin. The results presented represent the average of four independent experiments.

RESULTS

Expression and Activation of Prethrombin-2$^{(148)}$. A mutant of bovine thrombin was prepared in which the insertion loop around Trp^{148} (residues 144−150) was replaced by the loop from bovine trypsin. This mutant thrombin was overexpressed as a single-chain precursor, prethrombin-2, in a T7 expression system (pGEMEX-2). The yields of protein isolated as inclusion bodies were equivalent to those from wild-type prethrombin-2. Prethrombin-2$^{(148)}$ was refolded from a sulfonated form using oxidized and reduced glutathione as the redox couple. Complete activation of prethrombin-2$^{(148)}$ to thrombin$^{(148)}$ by *E. carinatus* snake venom was determined by silver staining of an SDS gel, with results similar to those shown in Figure 3 of DiBella *et al.* (1995). After purification by heparin affinity chromatography, activation to thrombin, and further purification, the yield of thrombin$^{(148)}$ was nearly identical to that achieved for the wild-type protein (0.7−0.9 mg/L).

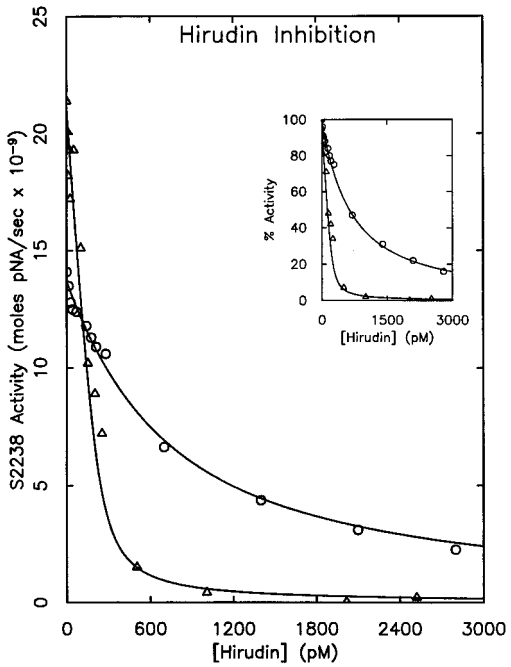

FIGURE 3: Inhibition of thrombin$^{(148)}$ and wild-type thrombin by hirudin. The inhibition of thrombin$^{(148)}$ and wild-type thrombin was monitored by the hydrolysis of S2238. Thrombin$^{(148)}$ (○) or thrombin (△) (0.2 nM) in 50 mM Tris, 0.15 M NaCl, 0.1% PEG, and 40 mg/mL BSA, pH 7.4, was incubated with various concentrations of hirudin (10 pM to 3 nM) at 37 °C. S2238 was added to 9.2 μM and the release of *p*-nitroaniline was followed at 405 nM. Curves represent the nonlinear least-squares fit of the data to eq 2. The data shown are from a representative experiment. Inset: The same data, except that they are normalized with S2238 activity in the absence of hirudin taken to be 100%.

The molecular mass of sulfonated prethrombin-2$^{(148)}$ was determined to be 35 863 ± 213 Da and of thrombin$^{(148)}$ to be 34 777 ± 37 Da by MALDTOF mass spectrophotometry. These experimentally determined values were equivalent to the calculated masses of 35 777 Da for sulfonated prethrombin-2$^{(148)}$ and 34 610 Da for thrombin$^{(148)}$. N-Terminal amino acid sequencing found the expected sequence of Ala-Ile-Glu-Gly-Arg-Thr-Ser-Glu-Asp-His-Phe-Gln for prethrombin-2$^{(148)}$.

Kinetic Characterization of Recombinant Thrombin$^{(148)}$. Thrombin$^{(148)}$ was compared to recombinant thrombin in terms of the ability to cleave fibrinogen (Table 1). For the release of FpA from fibrinogen, a k_{cat}/K_m of 4.5 ± 0.5 μM^{-1} s^{-1} was determined for thrombin$^{(148)}$ and 25 ± 2.0 μM^{-1} s^{-1} for wild-type thrombin. Thrombin$^{(148)}$ was found to have

Table 1: Comparison of Thrombin Activities on Fibrinogen[a]

	fibrinogen clotting (NIH units/mg)	FpA release k_{cat}/K_m (μM^{-1} s^{-1})
thrombin	2250 ± 250	25 ± 2.0
thrombin$^{(148)}$	1760 ± 150	4.5 ± 0.5

[a] Clotting times and k_{cat}/K_m for the release of FpA were determined as outlined under Materials and Methods. The results shown are an average of three indepedent experiments.

Table 2: Steady-State Kinetics for the Release of *p*-Nitroaniline by Recombinant Thrombin, Thrombin$^{(148)}$, and Trypsin[a]

substrate, enzyme	K_m[b] (μM)	k_{cat}[c] (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
S2238 (thrombin substrate)			
thrombin	2.9	123	42
thrombin$^{(148)}$	30	116	3.9
trypsin	41	70	1.7
S2222 (trypsin substrate)			
thrombin	113	2.1	0.019
thrombin$^{(148)}$	930	1.1	0.0012
trypsin	46	61	1.3
CbzGPRpNA			
thrombin	77	119	1.5
thrombin$^{(148)}$	422	84	0.2
trypsin	36	116	3.2

[a] Kinetic constants for the hydrolysis of the *p*-nitroaniline substrates were determined as described under Materials and Methods. The results shown represent an average of three independent experiments. [b] For K_m the standard errors were less than 10% for thrombin$^{(148)}$ (less than 5% for trypsin and thrombin). [c] For k_{cat}, the standard errors were less than 10%.

~75% the fibrinogen clotting activity (1760 NIH units/mg) of wild-type thrombin (2550 NIH units/mg).

To determine if the Trp^{148} loop replacement had imposed more trypsin-like activity on thrombin, the relative kinetics for the hydrolysis of different chromogenic peptide substrates were compared. The values of K_m and k_{cat} for S2238, S2222, and CbzGPRpNA were determined for thrombin, trypsin, and thrombin$^{(148)}$ (Table 2). S2238 should demonstrate the relative amount of thrombin activity remaining in thrombin$^{(148)}$. S2222, which is a good trypsin substrate but poor thrombin substrate, should demonstrate whether any further trypsin-like activity has been gained by the loop replacement. CbzGPRpNA, a substrate with comparable kinetics for both trypsin and thrombin, was used as a control.

The mutation of the Trp^{148} loop caused a 10-fold increase in the K_m value for the thrombin substrate, S2238 (from 2.9 to 30 μM). In contrast, the K_m for the hydrolysis of S2238 by trypsin was 41 μM. The k_{cat} for S2238 remained unchanged with the loop mutation (thrombin, 123 s^{-1}, vs thrombin$^{(148)}$, 116 s^{-1}) and was greater than the value obtained for trypsin (70 s^{-1}). The K_m determined for the hydrolysis of S2222 was found to be higher for thrombin$^{(148)}$ (930 μM) than for either wild-type thrombin (113 μM) or trypsin (46 μM). The same trend was found in the values of K_m for the hydrolysis of CbzGPRpNA (thrombin$^{(148)}$, 422 μM; thrombin, 77 μM; and trypsin, 36 μM). The k_{cat} determined for the hydrolysis of S2222 was 30−60-fold higher for trypsin (61 s^{-1}) than for either thrombin or thrombin$^{(148)}$ (2.1 s^{-1} and 1.1 s^{-1}, respectively). With CbzGPRpNA the k_{cat} determined was roughly the same with all three enzymes (119 s^{-1} for thrombin, 116 s^{-1} for trypsin, and 84 s^{-1} for thrombin$^{(148)}$). Hirudin, a potent inhibitor of thrombin, simultaneously binds close to the active site and at the fibrinogen anionic binding site. To examine binding at both of these sites, inhibition of S2238 activity by hirudin was determined for thrombin and thrombin$^{(148)}$ (Figure 3). Thrombin$^{(148)}$ was found to be 50% inhibited at 225 pM hirudin, whereas wild-type thrombin was 50% inhibited at 150 pM. Complete inhibition of thrombin$^{(148)}$ was not achieved even at 3 nM, whereas wild-type thrombin was 100% inhibited by hirudin at 1.0 nM. More specifically, the K_i for hirudin inhibition was determined to be ~40-fold higher with thrombin$^{(148)}$ (500 ± 120 pM) than with recombinant thrombin (12 ± 3 pM).

DISCUSSION

Thrombin is a serine protease with trypsin-like specificity for Arg−Xaa and Lys−Xaa bonds. While trypsin cleaves many of the arginyl and lysyl bonds in most proteins, only a few are substrates for thrombin. On the basis of the X-ray crystal structures of trypsin and PPACK-inhibited thrombin, it is apparent that the catalytic domains of the two proteins are remarkable similar (Bode *et al.*, 1992). One major difference in the two structures is the narrowing of the active-site cleft of thrombin by two insertion loops, one involving Trp^{148} and a second involving Trp^{60D}.

In order to understand the structural basis for the substrate specificity of thrombin, a mutant of thrombin was constructed in which the Trp^{148} loop was replaced with the equivalent loop from trypsin. This replacement has been shown to affect, but not abolish, the activity of thrombin. While thrombin$^{(148)}$ stills clots fibrinogen, hydrolyzes chromogenic substrates, and is inhibited by hirudin, the kinetic constants (K_m and K_i) have been affected by the replacement of the Trp^{148} loop.

Binding of substrate at the active site of thrombin has been altered by the loop replacement, as reflected by an increase in K_m for hydrolysis relative to wild-type thrombin for the substrates, S2238 (a thrombin substrate), S2222 (a trypsin substrate), and CbzGPRpNA (Table 2). For the hydrolysis of S2238, trypsin and thrombin$^{(148)}$ have approximately the same value of K_m, which is 10-fold higher than that for thrombin. In contrast, the K_m values determined for the hydrolysis of S2222 and CbzGPRpNA were 10−20-fold lower with trypsin than with thrombin$^{(148)}$ and 2-fold lower than with thrombin. These results demonstrate that the replacement of the Trp^{148} loop with the equivalent loop from trypsin has not produced an enzyme with more trypsin-like binding characteristics. In fact, the values of K_m found indicate an involvement of the Trp^{148} loop in the affinity of substrates for the active site of thrombin.

In contrast to the binding of substrates, the catalytic turnover of peptide substrates has not been significantly disrupted by this loop replacement. Similar k_{cat} values were determined with thrombin$^{(148)}$ and wild-type thrombin with all three substrates (Table 2). The release of FpA from fibrinogen examines catalysis as well as binding at both the active site and fibrinogen binding exosite of thrombin. A decrease in the determined value of k_{cat}/K_m for the release of FpA from fibrinogen was found (Table 1) for thrombin$^{(148)}$ compared to recombinant thrombin. The fact that the catalytic turnover of peptide substrates has not been altered suggests that the decrease in k_{cat}/K_m observed for the release of FpA is due to an increase in K_m and not a decrease in k_{cat}. This increase in K_m may arise from weaker binding

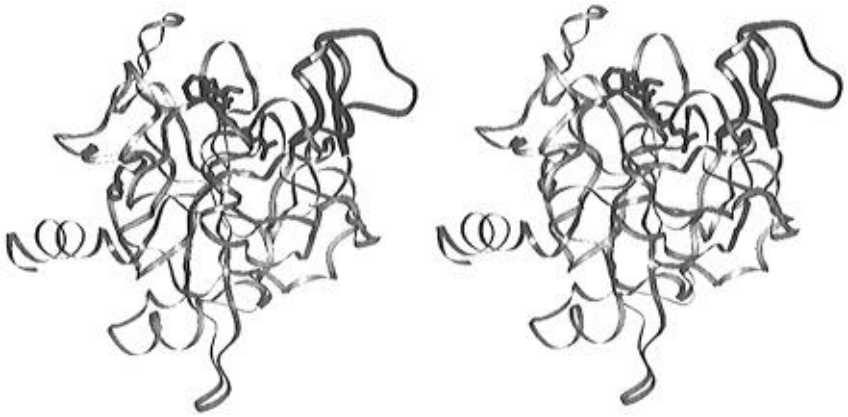

FIGURE 4: Stereo ribbon diagram showing a comparison of the Trp^{148} loop structure in PPACK-inhibited thrombin with its trypsin homolog. The PPACK moiety is shown as a black stick model at the top center of the structure. The Trp^{148} loop of thrombin, residues 144−151 (black line in the upper right-hand corner), was replaced by the trypsin homolog (alternating black and white segments). The structure of the trypsin loop was determined with a new protein modeling ECEPP package (J. Y. Trosset and H. A. Scheraga, to be submitted). Briefly, the trypsin loop was anchored in an extended conformation on human PPACK-inhibited thrombin. The ECEPP energy of the loop was then minimized in successive steps while keeping the rest of the protein fixed (J. Y. Trosset, E. E. DiBella, and H. A. Scheraga, to be submitted). A constraint energy allowed for the closure of the loop. At the end of the procedure, the *whole* structure was refined by energy minimization.

either at the active site, as with the peptide substrates, or at the fibrinogen anionic binding site. Since the magnitude of change in K_m is approximately the same for the hydrolysis of the peptide substrates as for the release of FpA, the observed difference in binding affinities between thrombin$^{(148)}$ and thrombin may be attributed to structural changes near the active site and not the fibrinogen binding anionic exosite.

Hirudin is a potent inhibitor of thrombin with a dissociation constant of 200 fM for human thrombin (Markwardt, 1970; Stone & Hofsteenge, 1986) and 20 pM for bovine thrombin (Dodt *et al.*, 1988). The N-terminus of hirudin binds at the active site of thrombin, without occupying the primary specificity site, and the last 16 C-terminal residues bind at the fibrinogen anionic binding site (Vitali *et al.*, 1992). The decreased affinity of hirudin for thrombin$^{(148)}$ (Figure 3) again demonstrates that the mutation of the Trp^{148} loop has likely altered binding near the primary binding site but does not rule out additional changes near the fibrinogen anionic exosite.

These kinetic findings are similar to the results found with a mutant in which residues Glu^{146}Thr^{147}Trp^{148} have been deleted from human thrombin (des-ETW thrombin) (Le Bonniec *et al.*, 1992). Higher values of K_m were determined with this mutant for S2238 and XaaProArgpNA substrates than for wild-type thrombin, but k_{cat} values were similar. The magnitude of the increase in K_m for des-ETW thrombin was 40−60-fold relative to wild-type thrombin, whereas this increase was only 6−10-fold for thrombin$^{(148)}$. The K_i for the inhibition of thrombin by hirudin was 30−100-fold higher for des-ETW thrombin relative to plasma or recombinant thrombin, which is similar to the finding of a 40-fold higher K_i for thrombin$^{(148)}$ when compared with wild-type thrombin. Our proposal that mutation of the Trp^{148} loop results in weaker binding affinity near the active site agrees with the results for des-ETW thrombin, irrespective of possible changes at the fibrinogen anionic exosite.

To determine whether replacement of the Trp^{148} loop has caused significant changes in the thrombin structure at the site of the replacement or elsewhere in the structure, modeling studies have been initiated. The Trp^{148} loop in the PPACK-inhibited thrombin crystal structure was replaced by the equivalent loop in trypsin. The structure of the trypsin loop in the PPACK-inhibited thrombin structure was determined with a new protein modeling ECEPP package (J. Y. Trosset and H. A. Scheraga, to be submitted). The trypsin loop was anchored on PPACK-inhibited thrombin in an extended conformation and the ECEPP energy minimized in successive steps while keeping the rest of the protein fixed (J. Y. Trosset, E. E. DiBella, and H. A. Scheraga, to be submitted). A constrained energy term allows for the closure of the loop; then, the *whole* structure of the mutant PPACK−thrombin was energy minimized in the absence of any constraints. The accommodation of the trypsin loop was checked by comparing the final optimized structure of the mutant PPACK−thrombin with the X-ray structure of wild-type PPACK−thrombin. The difference, as measured by the root-mean-square deviation of all the heavy atoms between the two structures, is 1.3 Å. On the basis of this modeling study, the replacement of the Trp^{148} loop with the equivalent trypsin loop can be accommodated without causing any significant disruption in the overall protein structure (Figure 4). More detailed modeling studies on the effect of the replacement of the Trp^{148} loop on the structure of PPACK-inhibited thrombin are in progress (J. Y. Trosset, E. E. DiBella, and H. A. Scheraga, to be submitted).

In the X-ray crystal structure of PPACK-inhibited human thrombin, there is one salt bridge involving a residue from the Trp^{148} loop. Glu^{146} forms a salt bridge with Arg^{221A} with an electrostatic energy of −1.4 kcal/mol (Bode *et al.*, 1992). A mutant of thrombin in which Arg^{221A} was changed to Ala was found to have reduced S2238 activity and retained <5% fibrinogen clotting activity relative to wild-type thrombin (Tsiang *et al.*, 1995). These findings indicate that the Glu^{146}−Arg^{221A} salt bridge may be involved in the regulation of the substrate specificity of thrombin.

In thrombin$^{(148)}$, the Glu^{146}−Arg^{221A} salt bridge has been removed by the deletion of Glu^{146}. To determine if the decrease in activity of thrombin$^{(148)}$ compared to wild-type thrombin is due to the removal of the Glu^{146}−Arg^{221A} salt bridge, $\Delta(\Delta G)$ was calculated from an estimate of the K_d values (k_{-1}/k_1) for the binding of S2238. $\Delta(\Delta G)$ is the difference in ΔG values for the dissociation of S2238 from thrombin$^{(148)}$ and wild-type thrombin, respectively.

Steady-state measurements of peptide substrate hydrolysis can be analyzed by the widely accepted mechanism of action of serine proteases (eq 4), where E, S, and P represent thrombin, substrate, and substrate cleavage products, respectively (Fersht, 1985).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \quad (4)$$

with

$$K_m = \frac{k_3(k_{-1} + k_2)}{k_1(k_3 + k_2)} \quad (5)$$

The individual k values for the hydrolysis of S2238 have been determined at pH 8.0 and 0.2 M NaCl by Wells and Di Cera (1992). From their experimental data, $k_3 \cong k_{-1}$ and, as a result, $K_d \cong K_m$. Therefore

$$\Delta(\Delta G) = -RT \ln(K_m^{mut}/K_m^{wt}) \quad (6)$$

where wt is wild-type thrombin and mut is thrombin$^{(148)}$. Using this approximation and the values of K_m from Table 2, a value of $\Delta(\Delta G) = -1.7$ kcal/mol is determined. We assume that this can be attributed to the electrostatic energy for the Glu^{146}−Arg^{221A} salt bridge, which has been estimated to be −1.4 kcal/mol by Bode *et al.* (1992). The changes in binding of thrombin$^{(148)}$ to peptide substrates, hirudin, and fibrinogen could, therefore, be due to the loss of the Glu^{146}−Arg^{221A} salt bridge.

CONCLUSIONS

The replacement of the Trp^{148} loop of thrombin with the equivalent loop from trypsin has been shown to alter the binding affinities of substrates to thrombin. On the basis of modeling studies, this mutation would not be expected to make large conformational changes in the protein structure. While the replacement of the Trp^{148} loop has affected the specificity of thrombin, it did not yield an enzyme with more trypsin-like specificity. As demonstrated through the kinetics of hydrolysis of chromogenic peptide substrates, there are changes in K_m, i.e., in the binding at the active site of thrombin$^{(148)}$ relative to thrombin. In general, the loop replacement has produced an enzyme with weaker binding affinities for peptide substrates than thrombin. However, the catalytic turnover of thrombin has not been affected by the replacement of the Trp^{148} loop. The difference in the specificity constant for the release of FpA from fibrinogen between thrombin$^{(148)}$ and wild-type thrombin may also be explained by weaker binding of fibrinogen to thrombin$^{(148)}$ at the active site. Upon replacement of the Trp^{148} loop with that of trypsin, a salt bridge involving Glu^{146}−Arg^{221A} is deleted which may account for the differences in binding that are observed.

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