Role of Leu99 of Thrombin in Determining the P2 Specificity of Serpins†

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ABSTRACT: A recent study indicated that Tyr99 (chymotrypsin numbering) of factor Xa and Thr99 of activated protein C are S2 subsite residues that determine the P2 specificity of their substrates and inhibitors. To investigate the contribution of Leu99 to the P2 binding specificity of thrombin, three mutants of thrombin were prepared in which Leu99 was substituted with Tyr (L99Y), Thr (L99T), or Gly (L99G). Kinetic analysis indicated that antithrombin (AT with P2 Gly) inhibited thrombin L99Y, 14.1- and 5.5-fold slower than thrombin in the absence and presence of heparin, respectively. The L99Y mutation increased the stoichiometry of AT inhibition in the presence of heparin from ∼1.6 to ∼4.6, indicating that L99Y recognized AT as a substrate. The inhibition rates of L99T and L99G by AT, respectively, were 500.0- and 916.7-fold slower than thrombin in the absence of heparin but only 41.8- and 64.5-fold slower than thrombin in the presence of heparin. Resolution of the two-step reactions of AT with the mutant thrombins revealed that the impaired reactivities occurred in the second reaction step in which a non-covalent AT−thrombin encounter complex is converted to a stable, covalent complex. In reactions with protein C inhibitor (PCI with P2 Phe), L99Y was inhibited 3.5-fold slower than thrombin, L99T was inhibited at a similar or faster rate, and L99G was inhibited 23.9-fold faster than thrombin. The epidermal growth factor-like domains 4−6 of thrombomodulin (TM4−6) accelerated the PCI inhibition of wild-type and L99G thrombins 73.9- and 5.3-fold, respectively. Further studies indicated that the fibrinogen clotting and protein C activation rates by the mutants were impaired, but the cofactor function of TM was not affected as TM4−6 bound to wild-type [$K_{d(app)}$ = 5.9 nM] and mutant thrombins with similar affinities [$K_{d(app)}$ = 4.4−6.9 nM] and enhanced protein C activation rates by all mutants effectively. These results indicate that (1) Leu99 of thrombin is critical for determination of the P2 specificity of serpins, AT and PCI, (2) increasing the polarity of the S2 pocket of thrombin by introduction of a hydrophilic residue into this pocket is detrimental for reaction with AT, but it is tolerated in reaction with PCI, so that only the size of the S2 pocket of thrombin determines the P2 specificity of PCI, and (3) the thrombomodulin-induced conformational change that results in acceleration of thrombin inhibition by PCI involves Leu99.

Thrombin is the terminal enzyme of the coagulation cascade which clots fibrinogen (Stubbs et al., 1992; Walz et al., 1986), activates platelets (Bevers et al., 1991), and up-regulates its own production by activating cofactors V and VIII (Mann et al., 1988). Thrombin also shuts down its own production when it binds thrombomodulin (TM)[1] on the surface of endothelial cells to activate protein C (Esmon, 1989). Activated protein C (APC) inactivates the active forms of cofactors V and VIII by limited proteolysis down-regulating thrombin generation in plasma (Vehar & Davie, 1980; Walker et al., 1979). Thrombin activity in plasma is also regulated by several serine protease inhibitors (serpins) including antithrombin (Damus et al., 1973), heparin cofactor II (Pratt & Church, 1993), protease nexin I (Stone & Hermans, 1995), and probably protein C inhibitor (PCI) (Rezaie et al., 1995).

Thrombin is a trypsin-like serine protease which recognizes its peptide substrates after basic residues, largely Arg but also Lys (Stubbs & Bode, 1993). Unlike trypsin, however, thrombin is a very specific protease which cleaves only after a limited number of peptide bonds. Although all determinants of thrombin specificity are not well understood, two major structural features appear to play key roles in molecular mechanisms by which thrombin recognizes its macromolecular substrates and inhibitors with a high degree of specificity. The first is that thrombin contains two surfaces rich in positively charged residues, referred to as anion binding exosites 1 and 2 (Stubbs & Bode, 1993). Binding of different cofactors to these exosites modulate thrombin function by allosteric mechanisms. The second is that there are several variant residues and insertion loops in the extended substrate binding pocket of thrombin which are unique for the structure of thrombin (Bode et al., 1992; Stubbs & Bode, 1993). The variant residues by virtue of their size, charge, and/or polarity make favorable or unfavorable interactions with the residues surrounding either side of the scissile bonds (particularly with the P3−P3′ residues)[2] of substrates and inhibitors, conferring a restricted specificity for thrombin.

In the X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited thrombin, the P2 Pro of the inhibitor fits into a hydrophobic pocket formed by the side chains of

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[1] Abbreviations: L99Y, L99T, and L99G, thrombin mutants in which Leu99 in the chymotrypsin numbering system of Bode et al. (1989) is substituted with Tyr, Thr, or Gly, respectively; AT, antithrombin; PCI, protein C inhibitor; serpin, serine protease inhibitor; DFP, diisopropyl fluorophosphate; APC, activated protein C; GDPC, Gla-domainless protein C; TM, thrombomodulin; PEG, polyethylene glycol.

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Trp215, Leu99, His57, Tyr60a, and Trp60d (Stubbs & Bode, 1993). In addition to the latter two residues which are unique to the structure of thrombin, Leu99 is another variant residue in the active site pocket of thrombin and other serine proteases. Residue 99 is Tyr in factor Xa and Thr in APC. Recent mutagenesis studies indicated that the exchange of this residue between factor Xa and APC switches the specificity of the mutant enzymes in their reactions with antithrombin. The mutant of factor Xa with a Thr at this position became resistant to inhibition by antithrombin and the mutant of APC with a Tyr at this position became susceptible to inhibition (Rezaie, 1996a). To determine the contribution of Leu99 to the specificity of thrombin, three mutants of thrombin were prepared in which this residue was replaced with a Tyr, Thr, or Gly. These mutants were expressed in mammalian cells as prethrombin-1 zymogens, and after purification and activation, their specificity profiles were studied with respect to activation of protein C, clotting of fibrinogen, and reactivities with antithrombin and PCI in the absence and presence of cofactors, heparin and TM. The results suggest that both the size and polarity of the S2 pocket are critical for optimal reaction of thrombin with protein C, fibrinogen, and antithrombin, but in reaction with PCI, only the size of this pocket dictates specificity. The results further indicate that TM accelerates PCI inhibition of thrombin by inducing a conformational change in the active site pocket of thrombin that involves Leu99.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of Recombinant Proteins. The expression and purification of recombinant wild-type thrombin has been previously described (Rezaie, 1996b). The L99Y, L99T, and L99G mutants of thrombin were prepared by standard polymerase chain reaction mutagenesis methods as described (Higuchi et al., 1988). The mutations were confirmed by DNA sequencing (Sanger et al., 1977). All manipulations were carried out in the prethrombin-1 derivative of prothrombin cDNA and then transferred to the pNUT-PL2 mammalian expression vector as described (Rezaie, 1996b). This vector codes for a mouse metallothionein promoter, a transferrin signal peptide, and a 12-residue HPC4 monoclonal antibody-binding epitope that permits Ca^{2+}-dependent purification on an HPC4 antibody column as described (Rezaie et al., 1992). This vector also contains a mutant *dhfr* gene for selection in a high concentration of methotrexate. The expression vector containing the prethrombin-1 cDNA fragment or its mutant derivatives was transferred to baby hamster kidney (BHK) cells by Lipofectin (GIBCO BRL, Gaithersburg, MD), and methotrexate resistant clones were selected and grown in a 96-well plate. Supernatants were examined for expression by an ELISA using prethrombin-1 specific polyclonal antibodies and the HPC4 monoclonal antibody. The expression level for all recombinant prethrombin-1 derivatives exceeded 5 μg/mL of cell culture supernatant.

[2] Nomenclature of Schechter and Berger (1967) used to describe the subsites of interaction between a protease and its substrate. Amino acid residues of the substrate are referred to as P1, P2, etc. on the N-terminal side of the substrate scissile bond, and those on the C-terminal side are referred to as P1′, P2′, etc. The corresponding sites on the enzyme where substrate residues interact are designed S1, S2, ... and S1′, S2′, ..., respectively.

Prethrombin-1 activation by the prothrombinase complex and purification on an FPLC Mono S column (Pharmacia LKB) were previously described (Ye et al., 1994). The concentration of recombinant thrombin derivatives were determined by absorbance at 280 nm, assuming a molecular weight of 36 600 and extinction coefficients ($E^{1\%}_{1cm}$) of 17.1 for thrombin and L99G and 17.5 for L99Y. The extinction coefficients for the mutants were calculated as described (Pace et al., 1995). The active-site concentration of recombinant thrombin derivatives was also determined as described (Mann et al., 1990) using BioCap-FPR-CK (biotinyl-ϵ-aminocaproyl-D-phenylalanine prolylarginine chloromethyl ketone; Haematologic Technologies Inc., VT) as the active site probe. The concentration of active enzyme determined by this method agreed within 10% of the values calculated on the basis of absorbance at 280 nm.

Bovine antithrombin was prepared as described (Kurachi et al., 1976; Owen, 1975). Recombinant TM4−6 was prepared as described (Rezaie & Esmon, 1992). Recombinant PCI (a generous gift from Dr. Frank Church, University of Chapel Hill, NC) was prepared as described (Cooper & Church, 1995). All proteins were homogeneous as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS−PAGE). Spectrozymes TH (SpTH) and PCa (SpPCa) were purchased from American Diagnostica (Greenwich, CT). The chromogenic substrates S2266 and S2238 were purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). Unfractionated heparin (porcine intestinal mucosa, sodium salt, grade II), polybrene, and hirudin were purchased from Sigma. α1-Antitrypsin was purchased from Athens Research and Technology, Inc. (Athens, GA).

Hydrolysis of p-Nitroanilide Chromogenic Substrates. The steady-state kinetic analysis of the hydrolysis of several commercially available substrates by the wild-type and the mutant enzymes was performed at room temperature in 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl (TBS), containing 1 mg/mL bovine serum albumin (BSA) and 0.1% polyethylene glycol 8000 (PEG 8000). The concentration of chromogenic substrates ranged from 1 to 2000 μM, and the concentration of enzymes ranged from 0.5 to 5 nM, depending on the k_{cat} values.

Inhibition Kinetics. The rates of inactivation of thrombin derivatives by antithrombin and PCI were measured under pseudo-first-order rate conditions. Both the inhibition reaction and the determination of residual thrombin activity were done in 96-well vinyl plates at room temperature in TBS buffer (ionic strength 0.12) containing 1 mg/mL BSA and 0.1% PEG 8000. Each thrombin derivative was incubated with at least a 10-fold molar excess of each serpin at room temperature for a period of time, SpPCa was then added to give a final concentration of 0.4 mM. The remaining chromogenic substrate activities of thrombin derivatives were measured with a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The second-order association rate constant (k_{assn}) of inhibition was calculated as described (Rezaie et al., 1995), using the equation

$$k_{assn} = (-\ln a)/t[I] \quad (1)$$

where a is residual protease activity, t is the time of the inhibition reaction, and [I] is the serpin concentration. In all experiments it was ensured that less than 10% of the chromogenic substrate was utilized and that all inhibition

Table 1: Steady-State Kinetics of Chromogenic Substrate Hydrolysis by Wild-Type and Mutant Thrombins[a]

	SpTH			SpPCa			S2266			S2238		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
thrombin	4.2	17.1	4.1	4.9	21.4	4.4	221.5	8.8	0.04	7.6	31.0	4.1
L99Y	54.0	31.8	0.6	35.7	53.4	1.5	127.1	11.8	0.09	24.0	44.1	1.8
L99T	321.4	77.1	0.2	39.0	43.1	1.1	439.6	17.0	0.04	34.8	45.1	1.3
L99G	32.4	6.2	0.2	6.5	15.1	2.3	186.9	5.3	0.03	6.3	1.1	0.2

[a] The values of the kinetic constants K_m (μM), k_{cat} (s^{-1}), and K_m/k_{cat} (μM^{-1} s^{-1}) for each chromogenic substrate were determined in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 as described under Materials and Methods. All values are the average of at least three independent measurements with ±SD values of 7% or less for the K_m determinations and 10% or less for the k_{cat} determinations.

assays were performed by time course analysis to obtain at least 50% enzyme inhibition for calculation of inhibition rates.

The rate of PCI inactivation of each thrombin mutant was also measured in the presence of cofactors, heparin and TM4−6. In this case each thrombin derivative (0.5 nM) was incubated with a 20-fold excess of PCI (10 nM) at room temperature with 1 U/mL heparin (determined to be the optimal concentration) or with saturating concentration of TM4−6 (100 nM) in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000, as previously described (Rezaie et al., 1995). After a period of time (30 s to 2 min), SpPCa in TBS buffer containing 1 mg/mL polybrene (to stop heparin action) was added to final concentration of 0.4 mM, the remaining activities of uninhibited thrombins were measured, and the k_{assn} values were calculated as described above.

The rate of inactivation of thrombin and the mutants in the presence of heparin was measured under pseudo-first-order rate conditions by a continuous assay method in the presence of SpTH as the competing chromogenic substrate as described previously (Griffith, 1982). In this assay, 0.5−1 nM thrombin was incubated with 25−50 nM antithrombin, 1 unit/mL heparin (determined to be an optimal concentration for all of the thrombin derivatives), and 250 μM SpTH in a 96-well vinyl plate. The hydrolysis of SpTH was monitored at 10 s time intervals immediately after thrombin addition. The apparent pseudo-first-order rate constant of inhibition was estimated by fitting the absorbance at 405 nm versus time into the following equation:

$$A = a_o/k_{obs}(1 - e^{-k_{obs}t}) + A_o \quad (2)$$

where t is the time of the inhibition, A is the absorbance at 405 nm at time t, A_o is the absorbance at 405 nm at time zero, a_o is the thrombin activity at time zero, and k_{obs} is the apparent pseudo-first-order rate constant of inhibition. To correct for the presence of chromogenic substrate, the pseudo-first-order rate constant of inhibition k' was given by

$$k' = k_{obs}(1 + [S]/K_m) \quad (3)$$

where [S] is the concentration of the chromogenic substrate, SpTH, and K_m is the Michaelis−Menten constant of the thrombins for SpTH. The K_m values for hydrolysis of SpTH by all proteases are listed in Table 1. The second-order association rate constants were calculated from the ratio of k' to antithrombin concentration.

The inhibition of thrombin mutants by antithrombin were also studied by the slow-binding kinetic approach as described (Morrison & Walsh, 1988). In this case, a series of inhibition progress curves for each derivative were generated by adding 0.5 nM enzyme (final concentration) to wells of a 96-well plate containing various concentrations of antithrombin (12.5−500 nM) and 1 unit/mL heparin and 150−250 μM SpTH (for wild-type, L99Y, and L99G thrombins) or 150 μM SpPCa (for L99T) were used as the competing chromogenic substrates. To ensure that the concentration of heparin was not rate-limiting in the assays, the concentration dependence of the heparin acceleration of L99T and L99G inhibition was determined with 500 nM antithrombin (the highest concentrations of antithrombin used in these assays). Heparin at 1 unit/mL was found to be the optimal heparin concentration in these assays, as well. In the absence of heparin, the inhibition rates were studied under the same conditions except that 12.6−75.5 μM antithrombin and 300 μM S2238 (for thrombin) or 400 μM S2266 (for L99Y) were used as the competing chromogenic substrates. In the absence of heparin by this method of analysis no values were determined for L99T and L99G thrombins. With L99T and L99G thrombins, antithrombin concentrations as high as 75.5 μM did not result in a significant inhibition of the enzymes activities even with a low concentration (100 μM) of the competing chromogenic substrate, S2266, which has a high K_m value for these mutant thrombins (Table 1). Data from each curve at different inhibitor concentrations were fit by nonlinear regression analysis to the integrated rate equation for slow-binding inhibition (Morrison & Walsh, 1988).

$$A = v_s t + (v_o - v_s)(1 - e^{-k_{obs}t})/k_{obs} + A_o \quad (4)$$

where A is absorbance at 405 nm at time t, v_o and v_s are initial and final steady-state velocities, respectively, k_{obs} is the apparent first-order rate constant, and A_o is the initial absorbance at 405 nm. Fitting estimates values for v_o, v_s, k_{obs}, and A_o for each progress curve. In all cases only the data with less than 10% chromogenic substrate hydrolysis were analyzed.

For determination of the rate constants for reaction with diisopropylfluorophosphate (DFP), 2−4 nM of each thrombin derivative was added to dilutions of DFP from 7.5 to 500 μM in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000 at room temperature. The reactions were monitored for 30 min by time course analysis. The remaining activities were determined by an amidolytic activity assay using chromogenic substrate as described above. The rate constants were measured from the first-order rate equation as described above.

Determination of Antithrombin Inhibition Stoichiometry. The SI values for antithrombin inhibition of thrombin derivatives were determined by titration of 100 nM thrombin in the presence of 2 units/mL heparin or 200 nM thrombin

in the absence of heparin with increasing concentrations of antithrombin, corresponding to antithrombin/thrombin molar ratios of 0 to 5, in the TBS buffer system described above. The residual amidolytic activities of thrombin and the mutant enzymes were monitored for up to 24 h at room temperature by the hydrolysis of SpPCa as described above. After completion of the inhibition reactions, the antithrombin/thrombin ratios were plotted versus the residual activities of enzymes and the SI values were determined from the *x*-intercept of the linear regression fit of the plotted data.

The SI values of PCI inhibition of wild-type and mutant thrombins were determined by titration of 2 nM thrombin in the presence of 1 unit/mL heparin with increasing concentration of PCI, corresponding to PCI/thrombin molar ratios of 0 to 12 as described (Cooper & Church, 1995).

Analysis of Thrombin−Antithrombin Reaction Products by Gel Electrophoresis. Complex formation of thrombin or the mutants with antithrombin was monitored by gel electrophoresis. The reactions were carried out in 50 μL at room temperature with 2.5 μM thrombin derivatives and 2.5 μM antithrombin for 15 min in the absence of heparin and for 3 min in the presence of 5 units/mL heparin. After completion of the reactions, 10 μL of 5× non-reducing SDS sample buffer was added, the resulting solution was boiled for 5 min, and 10 μL of each reaction was analyzed on 10% SDS−PAGE gel stained with Coomassie Blue R-250 as described (Laemmli, 1970).

Protein C Activation. The initial rates of protein C activation and the affinities of wild-type and the mutant thrombins for thrombomodulin fragment 4−6 (TM4−6) were determined as described previously (Liu et al., 1994) except that recombinant Gla-domainless protein C (GDPC) was used as the substrate (Rezaie & Esmon, 1992).

Clotting Assays. The activity of wild-type and mutant thrombins were compared in clotting assays using human plasma or purified fibrinogen (Kabi Diagnostica, Stockholm, Sweden). Clotting assays with pure fibrinogen were done at fibrinogen concentrations well below the K_m for cleavage of this substrate by thrombin. Under such conditions the clotting time is limited by the rate of cleavage of fibrinogen by thrombin (De Cristofaro & Di Cera, 1991). Clotting was initiated by adding 0.3−2.5 nM wild-type, 1.25−15 nM L99T or L99G, and 31−125 nM L99Y thrombins to a solution of 0.25 μM fibrinogen in TBS buffer containing 2.5 mM $CaCl_2$, 0.1% PEG 8000, pH 7.5 at 22−25 °C. Clot formation was continuously monitored from the sigmoidal increase in absorbance at 405 nm due to light scattering until a plateau absorbance was reached. The clotting time was determined as the time corresponding to the intersection of a line drawn through the steepest part of the clotting curve with the base line absorbance (De Cristofaro & Di Cera, 1991). Human plasma clotting was initiated by addition of 100 μL of 2 units/mL thrombin (∼22 nM) in TBS buffer containing 2.5 mM $CaCl_2$ and 0.1% PEG 8000 to 100 μL of citrated human plasma at 37 °C, to give a final concentration of 1 unit/mL thrombin and the clotting time was measured with an ST4 Biocoagulometer (Diagnostica/Stago, Asnieres, France). Under these conditions a clotting time of 17.7 s was obtained for wild-type thrombin. The same conditions were used for fibrinogen clotting except that 100 μL of 6 mg/mL human fibrinogen in TBS buffer was used instead of human plasma. Under these conditions a clotting time of 17.4 s was obtained for wild-type thrombin. Both types of clotting assays were done with concentrations in the ranges 0.125−1 unit/mL (1.4−11 nM) wild-type, 0.75−6 units/mL (8.25−66 nM) L99T or L99G, and 12.5−100 units/mL (137.5−1100 nM) L99Y thrombins. Log−log plots of clotting time vs thrombin concentrations were linear with indistinguishable slopes for all thrombins in all clotting assays.

Data Analysis. The K_m and k_{cat} values for substrate hydrolysis were calculated from the Michaelis−Menten equation, and the inhibition rate constants were calculated from the equations mentioned above using ENZFITTER computer program (R. J. Leatherbarrow, Elsevier, Biosoft). All of the data presented are the average of at least three to six independent measurements ± SD.

RESULTS

The prethrombin-1 forms of the mutant proteins were expressed in BHK cells to high yields and purified by single-step immunoaffinity chromatography using HPC4 monoclonal antibody immobilized on Affigel-10 as described under Materials and Methods. The purified proteins were essentially pure as determined by SDS−PAGE (data not shown). All prethrombin-1 derivatives were activated by the prothrombinase complex, and thrombins were purified by Mono S ion-exchange chromatography. On the Coomassie-stained SDS−PAGE gels, the recombinant thrombins appeared as two closely spaced migrating bands with the apparent molecular masses of ∼37 and ∼38 kDa. The 37 kDa band was the major protein in all recombinant thrombins except for L99Y thrombin in which both of these bands appeared at equal intensities, suggesting that the L99Y mutation is associated with a change in the ratio of the differentially glycosylated variants of thrombin (data not shown). Active site titration indicated that all enzymes had ≥90% of the anticipated active site concentrations.

To ensure that the mutations did not adversely affect the charge-stabilizing system and the reactivity of the catalytic triads, the DFP and hirudin inhibition profiles of wild-type and mutant thrombins were compared. DFP potently inactivates most serine proteases by irreversibly acylating the active-site residue, Ser195. DFP inactivated wild-type thrombin with a second-order association rate constant of 14.2 ± 2.1 M^{-1} s^{-1}. The same values for reaction with the mutants were 23.8 ± 3.9 M^{-1} s^{-1} for L99Y, 13.0 ± 1.5 M^{-1} s^{-1} for L99T, and 7.8 ± 1.8 M^{-1} s^{-1} for L99G thrombins. The observation that the rate constant was lower for L99G may suggest that the mutation either slightly affected the conformation of the catalytic Ser195, or the S2 subsite of thrombin also plays a role in thrombin reaction with DFP. The enhanced reactivity of L99Y thrombin with DFP is consistent with the latter hypothesis. With respect to inhibition by hirudin, incubation of 1 nM wild-type or mutant thrombins with 1 nM hirudin for 20 min at room temperature followed by addition of the chromogenic substrate, SpPCa, to concentrations equal to 10K_m value for each mutant, resulted in similar inhibition of the thrombin activity for all derivatives (57%, 55%, 56%, and 57% for wild-type, L99Y, L99T, and L99G thrombins, respectively). These results suggest that the folding or the reactivities of the active-site pockets are minimally affected by the mutations.

Chromogenic Substrate Specificity of Thrombin Mutants. Previous analysis of residue 99 substitution mutants of factor

Table 2: Second-Order Association Rate Constants for Antithrombin Inhibition of Wild-Type and Mutant Thrombins in the Absence and Presence of Heparin

	k_{assn} (M^{-1} s^{-1})[a] AT (-heparin)	k_{assn} (M^{-1} s^{-1})[b] AT (+heparin)	fold acceleration
thrombin	$(1.1 \pm 0.1) \times 10^4$	$(7.1 \pm 0.3) \times 10^6$	645
L99Y	$(7.8 \pm 0.2) \times 10^2$	$(1.3 \pm 0.2) \times 10^6$	1667
L99T	$(2.2 \pm 0.2) \times 10^1$	$(1.7 \pm 0.2) \times 10^5$	7727
L99G	$(1.2 \pm 0.2) \times 10^1$	$(1.1 \pm 0.1) \times 10^5$	9167

[a] The values are calculated using eq 1. [b] The values are calculated using eq 2.

Xa and APC (FXa Y99T/Q192E and APC T99Y) demonstrated that residue 99 determines the P2 specificity of chromogenic substrates for both of these proteases (Rezaie, 1996a). To examine the influence of Leu99 in the chromogenic substrate specificity of thrombin, the kinetic constants for the hydrolysis of selected chromogenic substrates by recombinant thrombin and the mutants were determined under steady-state conditions (Table 1). Both L99Y and L99T thrombins hydrolyzed SpTH, SpPCa, S2266, and S2238 better than wild-type thrombin at saturating concentrations of these substrates. As shown in Table 1, the k_{cat} constants for hydrolysis of all four chromogenic substrates were increased relative to thrombin. However, the increase in the k_{cat} constants was accompanied by an increase in the K_m values, resulting in an overall lower catalytic specificity (k_{cat}/K_m) for these mutant enzymes. With the exception of SpTH, L99G thrombin cleaved all chromogenic substrates with K_m values similar to those of thrombin, but the k_{cat} values were impaired with this mutant (Table 1).

Reaction with Serpins. The second-order association rate constants (k_{assn}) for antithrombin inhibition of thrombin and the mutants in the presence and absence of heparin are shown in Table 2. In the absence of heparin, antithrombin inhibited thrombin with $k_{assn} = (1.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1}. Under the same conditions, the rates of inhibition of L99Y, L99T, and L99G thrombins by antithrombin were 14.1-, 500.0-, and 916.7-fold slower than thrombin, respectively. Heparin accelerated the inhibition rates 645-fold with wild-type, 1667-fold with L99Y, 7727-fold with L99T, and 9167-fold with L99G thrombins.

Antithrombin inhibits thrombin by a two-step reaction mechanism in which an enzyme−inhibitor encounter complex is initially formed which is then converted to a stable, covalent complex (Olson & Shore, 1982). To determine whether the impaired rate of antithrombin inhibition of thrombin mutants relative to wild-type thrombin was due to an effect on the first or the second reaction step, the pseudo-first-order inhibition rate constants (k_{obs}) were determined from the slow-binding kinetic analysis for several concentrations of antithrombin. A saturable dependence of k_{obs} on antithrombin concentration was observed for inhibition of L99T and L99G thrombins in the presence of heparin (Figure 1) and for inhibition of L99Y thrombin in the absence of heparin (data not shown). Nonlinear least squares analysis of the hyperbolic saturation curves obtained for all reactions yielded the values for the ternary complex dissociation constant and the rate constant for stable complex formation shown in Scheme 1. The resolution of the two-step kinetic mechanism of thrombin−antithrombin reaction for L99T and L99G thrombins in the absence of heparin was not possible

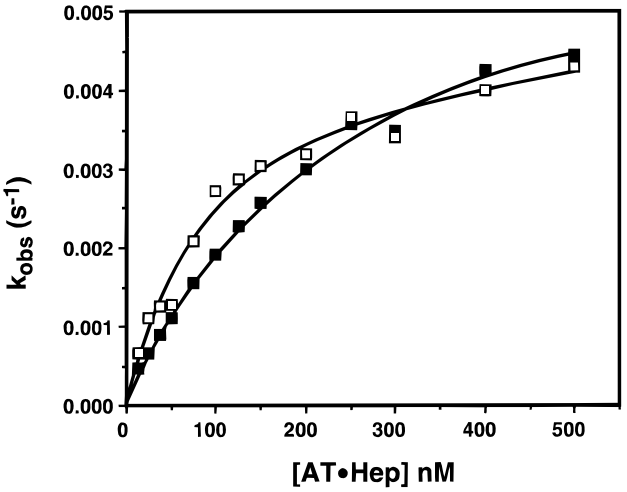


FIGURE 1: Dependence of k_{obs} on antithrombin concentration with L99T and L99G thrombins in the presence of heparin. The pseudo-first-order rate constants, k_{obs}, for inhibition of L99T (□) or L99G (■) thrombins were calculated from a series of inhibition progress curves using eq 4 as described in Materials and Methods. The equilibrium dissociation constants and the rate constants were estimated from nonlinear regression fit of data to a rectangular hyperbola. The values are shown in Scheme 1. The values for equilibrium dissociation constants in Scheme 1 are divided by $1 + [S]/K_m$ to correct for substrate competition.

Scheme 1

$$AT + T_{L99Y} \xleftrightarrow{23.4 \pm 1.8\ \mu M} AT{\bullet}T_{L99Y} \xrightarrow{0.015 \pm 0.001\ s^{-1}} AT{-}T_{L99Y}$$

$$AT{-}H + T_{L99T} \xleftrightarrow[\bullet H\bullet]{39.9 \pm 15.4\ nM} AT{\bullet}T_{L99T} \xrightarrow{0.006 \pm 0.002\ s^{-1}} AT{-}T_{L99T} + H$$

$$AT{-}H + T_{L99G} \xleftrightarrow[\bullet H\bullet]{26.2 \pm 7.5\ nM} AT{\bullet}T_{L99G} \xrightarrow{0.005 \pm 0.001\ s^{-1}} AT{-}T_{L99G} + H$$

by this method of analysis since antithrombin concentrations as high as 75.5 μM in the presence of 100 μM S2266 resulted in less than 10% inhibition of the enzyme activity. It was previously demonstrated (Rezaie, 1996b) that this simple kinetic approach of using chromogenic substrates has not been successful for resolution of the wild-type thrombin−antithrombin reaction steps in either the absence or the presence of heparin. This has been due to the high initial K_D, as well as the high rate constant of the final stable complex formation (Olson & Shore, 1982) which prevents using high concentration of antithrombin in the reactions to observe saturation kinetics. This was particularly true with L99Y thrombin, since not only was the rate constant high in the presence of heparin but also the K_m values for competing chromogenic substrates were elevated for this mutant (Table 1). It was however, possible to resolve the reaction steps for L99Y thrombin in the absence of heparin (Scheme 1, top equation). Comparisons of the values obtained by the slow-binding kinetic methods here, with those previously determined by the rapid-kinetic methods for wild-type thrombin in the presence of heparin [$K_D = 140 \pm 20$ nM, and $k = 3.2 \pm 0.1$ s^{-1} (Rezaie & Olson, 1997)], indicate that, if anything, the K_D for the ternary encounter complex formation is improved with the mutants, therefore, the decreased rate of antithrombin inhibition results from a decreased rate constant for the formation of a stable, covalent complex. The second-order association rate constants, as determined from the ratio of the rate constants to K_D values (6.4×10^2 M^{-1} s^{-1} for L99Y in the absence of heparin, and

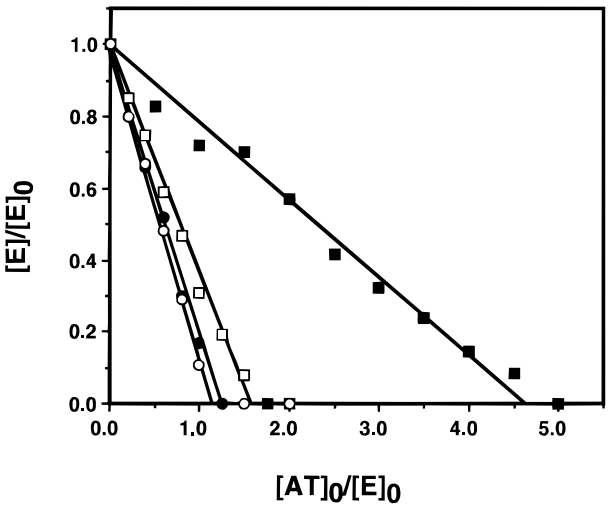


FIGURE 2: Determination of the stoichiometries of inhibition (SI) for the recombinant wild-type and mutant thrombins in the presence of heparin. Titration of fixed amount of each thrombin derivative (100 nM) with increasing concentration of antithrombin was monitored from the residual amidolytic activities as described under Materials and Methods. The symbols are (□) thrombin, (●) L99T, (○) L99G, and (■) L99Y. The solid lines are linear regression fits of the inhibition data.

1.5×10^5 M^{-1} s^{-1} for L99T, and 1.9×10^5 M^{-1} s^{-1} for L99G both in the presence of heparin), are in good agreement with the values calculated by the other methods for these mutants (Table 2), supporting the reliability of the slow-binding kinetic methods for the resolution of the two reaction steps in the thrombin mutants−antithrombin reactions.

To determine whether the mutations influenced the substrate pathway of the reaction with antithrombin, a series of experiments were performed to calculate the SI values of complex formation for the mutants in the absence or presence of heparin. In the absence of heparin it was only possible to determine the SI values for wild-type and L99Y thrombins, since the reactivities of L99T and L99G with antithrombin were severely impaired (Table 2). Antithrombin in the absence of heparin inhibited both wild-type and L99Y thrombins with a 1:1 stoichiometry (data not shown). In the presence of heparin the SI values were 1.6 ± 0.1 for wild-type, 4.6 ± 0.2 for L99Y, 1.2 ± 0.1 for L99T, and 1.1 ± 0.1 for L99G thrombins (Figure 2). The extent of complex formation between the thrombin mutants and antithrombin was monitored directly by SDS−PAGE analysis following incubation of an equimolar amount of each thrombin derivative with antithrombin in the absence or presence of heparin. As shown in Figure 3, in the absence of heparin wild-type thrombin formed high molecular weight complexes with antithrombin (lane 3). Under the same conditions, L99Y also formed high molecular weight complexes with antithrombin but at a lower efficiency (lane 4). On the other hand, consistent with the kinetic data very few high molecular weight complexes were observed with either L99T or L99G thrombins, suggesting that the reactivities of these mutants with antithrombin are severely impaired (lanes 5 and 6). In the presence of heparin in addition to forming high molecular weight complexes, thrombin also cleaved antithrombin, as evidenced by a decrease in the mobility of cleaved antithrombin on SDS−PAGE under non-reducing conditions (lane 7). This result is consistent with the literature (Olson, 1985). In the presence of heparin, however, very few high molecular weight complexes were observed

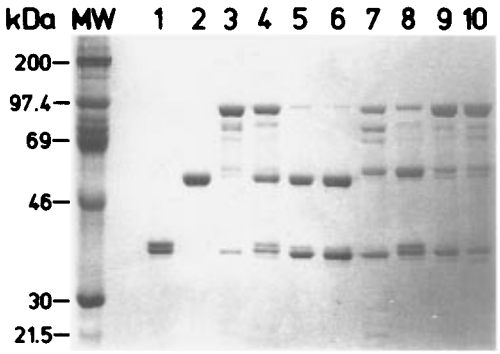


FIGURE 3: SDS−PAGE analysis of the stable thrombin−antithrombin complexes. Antithrombin (2.5 μM) was incubated with equimolar concentrations of thrombin for 15 min in the absence of heparin or 3 min in the presence of 5 units/mL heparin in 50 μL reactions in TBS buffer at room temperature. After addition of 10 μL of non-reducing sample buffer and boiling of samples, 10 μL of each inhibition reaction was loaded on 10% resolving gel under non-reducing conditions. The stable thrombin−antithrombin complexes migrated as the highest molecular weight bands, followed by slightly lower molecular weight bands representing the degraded complexes. Under non-reducing conditions, the reactive site cleaved antithrombin migrated slightly slower than native antithrombin as described under Results. Lane MW, protein standards; lane 1, thrombin L99Y; lane 2, native antithrombin; lanes 3−6, respectively, wild-type, L99Y, L99T, and L99G thrombins incubated with antithrombin in the absence of heparin; lanes 7−10, the same as lanes 3−6 except that thrombins are incubated with antithrombin in the presence of heparin.

with L99Y thrombin (lane 8). Consistent with an elevated SI value, L99Y thrombin cleaved antithrombin. With L99T and L99G thrombins, most of the antithrombin formed high molecular weight complexes (lanes 9 and 10). Although some amount of cleaved inhibitor was observed with these mutants, the relative amount of the cleaved inhibitor was less than that observed in the wild-type thrombin−antithrombin reaction. This is consistent with the reduced SI values for these mutants (Figure 2).

Inhibition by α1-Antitrypsin. The second-order association rate constants for α1-antitrypsin inhibition of thrombin derivatives were determined by methods similar to those described for antithrombin. The values were $(7.8 \pm 0.5) \times 10^1$ M^{-1} s^{-1} for wild-type, $(2.3 \pm 0.2) \times 10^1$ M^{-1} s^{-1} for L99Y, $(0.8 \pm 0.1) \times 10^1$ M^{-1} s^{-1} for L99T, and $(1.5 \pm 0.3) \times 10^1$ M^{-1} s^{-1} for L99G thrombins. Changing either the size or the polarity of the S2 pocket of thrombin was detrimental for reaction with α1-antitrypsin. These results are consistent with the structural data that Pro at the P2 position is the most preferred residue for fitting to the S2 pocket of thrombin (Stubbs & Bode, 1993). The slow reactivity of α1-antitrypsin with thrombin is due primarily to presence of a Met at the P1 position of the serpin which is not favored by thrombin or other trypsin-like serine proteases that exhibit a strong preference for Arg or Lys at this position. It should however, be noted that Glu192 of thrombin is also responsible for poor reactivity of thrombin with α1-antitrypsin since substitution of this residue with a neutral residue such as Gln or Met results in thrombin mutants that are rapidly inhibited by this serpin (Rezaie & Esmon, 1996).

Inhibition by Protein C Inhibitor. In contrast to reaction with antithrombin, the reactivity of PCI with both L99T and L99G thrombins was improved (Table 3). PCI inhibited L99T at a similar or faster rate than wild-type thrombin. In

Table 3: Second-Order Association Rate Constants for PCI Inhibition of Wild-Type and Mutant Thrombins in the Absence and Presence of TM4−6 or Heparin

	k_{assn} (M^{-1} s^{-1})			fold acceleration	
	PCI	PCI + TM4−6	PCI + heparin	TM4-6	heparin
thrombin	$(2.3 \pm 0.2) \times 10^4$	$(1.7 \pm 0.3) \times 10^6$	$(9.1 \pm 0.1 \times 10^5)$	73.9	39.6
L99Y	$(6.5 \pm 0.5) \times 10^3$	$(2.0 \pm 0.5) \times 10^5$	$(2.2 \pm 0.2) \times 10^5$	30.8	33.8
L99T	$(2.5 \pm 0.1) \times 10^4$	$(1.1 \pm 0.1) \times 10^6$	$(1.5 \pm 0.1) \times 10^6$	44.0	60.0
L99G	$(5.5 \pm 0.6) \times 10^5$	$(2.9 \pm 0.3) \times 10^6$	$(8.1 \pm 0.2) \times 10^6$	5.3	14.7

reaction with L99G, the rate of inhibition was improved 23.9-fold. PCI inhibited L99Y thrombin 3.5-fold slower than thrombin (Table 3). It is noteworthy that PCI also inhibits factor Xa at a similar slower rate (Pratt & Church, 1993), suggesting that L99Y thrombin is more like factor Xa with respect to inhibition by PCI. Heparin accelerated the inhibition rate of all mutants by PCI, and the cofactor function of heparin was the highest for L99T thrombin (Table 3). Interestingly, the effector function of heparin is also the highest with APC (Pratt & Church, 1993), suggesting that L99T thrombin is more like APC with respect to inhibition by PCI both in the absence and presence of heparin. The SI value for PCI inhibition of wild-type thrombin was ~4, which is similar to a previous report in the literature (Cooper & Church, 1995). The SI values were slightly elevated (SI $\approx$ 6) for all of the mutant thrombins (data not shown). A detailed study by the SDS−PAGE analysis was not possible for thrombin−PCI reactions due to depletion of the PCI reserve.

A previous study also showed that PCI inhibits thrombin rapidly in the presence of thrombomodulin (Rezaie et al., 1995). In the presence of a saturating concentration of TM4−6, PCI inhibited wild-type thrombin with a 73.9-fold higher rate constant (Table 3). For the mutants, the effect of TM4−6 was a 30.8-fold increase in the rate of L99Y inhibition and a 44.0-fold increase in the rate of L99T inhibition. The cofactor function of TM for inhibition of L99G was the lowest, as TM4−6 accelerated PCI inhibition of L99G thrombin only 5.3-fold. The inhibition of L99G thrombin by PCI was markedly faster than thrombin in the absence of TM4−6, but the inhibition rates were similar in the presence of TM4−6 (Table 3).

To ensure that the mutations did not impair the cofactor function of TM by adversely affecting the conformation or folding of the TM-binding exosite 1 of thrombin, the initial rates of protein C activation by the wild-type and mutant thrombins were determined as a function of different TM4−6 concentrations (Table 4). TM4−6 bound to the thrombin derivatives with similar $K_{d(app)}$ values of 5.9 ± 2.1 nM for wild-type, 4.4 ± 0.8 nM for L99Y, 6.9 ± 1.6 nM for L99T, and 6.0 ± 1.8 nM for L99G thrombins (n = 3, ±SD). Although all thrombin mutants activated protein C at a much lower rate than wild-type thrombin, nevertheless, the cofactor function of TM was improved in all cases, suggesting that the anion binding exosite 1 of thrombin is not perturbed as the result of the mutations (Table 4).

Similar to impairment observed in the activation of protein C, the mutant thrombins clotted fibrinogen slower than wild-type thrombin. Under conditions well below the K_m for fibrinogen where fibrinopeptide cleavage by thrombin is rate-limiting with respect to the subsequent aggregation of fibrin (De Cristofaro & Di Cera, 1991), the fibrinogen clotting times of L99G, L99T, and L99Y were ~25%, 20%, and 0.5% of wild-type thrombin, respectively. Similarly, the clotting time in a standard clotting assay using human plasma or pure fibrinogen at concentrations exceeding the fibrinogen K_m were ~20% for both L99G and L99T thrombins and ~0.7% for L99Y thrombin. The parallel plots of log of clotting time vs log of thrombin dilution obtained for wild-type and mutant thrombins in each assay indicated that these results were independent of thrombin dilution.

Table 4: Apparent Dissociation Constants ($K_{d(app)}$) for TM4−6 Binding and Gla-Domainless Protein C Activation by Wild-Type and Mutant Thrombins in EDTA or TM4−6 Plus Ca^{2+}

	$K_{d(app)}$ (nM) TM4−6	EDTA (mmol/min/mol)[a]	TM4−6 + Ca^{2+} (mmol/min/mol)	fold acceleration
thrombin	5.9 ± 2.1	78	825	10.6
L99Y	4.4 ± 0.8	0.35	15	42.9
L99T	6.9 ± 1.6	0.35	25	71.4
L99G	6.0 ± 1.8	0.19	13	68.4

[a] Activation rates are expressed as mol of activated GDPC generated/min/mol of thrombin with 2 μM substrate at room temperature.

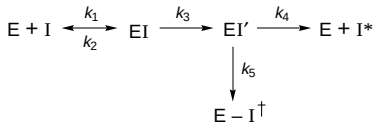
DISCUSSION

In this study, the contribution of Leu99 to the S2 specificity of thrombin in reaction with several chromogenic substrates, serpin inhibitors, and macromolecular substrates was examined by constructing three different mutants of thrombin in which Leu99 was substituted with Tyr, Thr, or Gly. The rational was that since the mutant residues all have different structures they could confer different properties to the S2 subsite of thrombin, potentially providing useful molecular probes for studying the factors that may influence the P2 binding specificity of thrombin. For instance, the side chain of Tyr is larger than Leu, and therefore it would further restrict the size of the S2 specificity pocket. Gly is a very small residue so it could create a larger S2 pocket, and Thr, on the other hand, is expected to increase the hydrophilicity of this pocket. If folded correctly, these mutants could provide valuable information about the role of size and/or polarity of the S2−P2 interactions in restricting the specificity of thrombin. Kinetic analyses with several chromogenic substrates (Table 1) indicated that both L99Y and L99T thrombins cleaved these substrates with higher k_{cat} values, suggesting that the mutations probably did not cause any deleterious conformational change to impair the charge stabilizing system or the reactivity of the catalytic triads. This is also supported by improved or similar reactivities of these mutants with DFP. Similar to wild-type thrombin, the L99G mutant exhibited a low K_m value for these substrates (except for SpTH), although the k_{cat} values for hydrolysis of these chromogenic substrates were impaired with this mutant. L99G thrombin also reacted at slightly lower rate than wild-type thrombin with DFP (1.8-fold slower). This may not however, suggest that the active-site

Ser195 is adversely affected, since the reactivity of DFP with L99Y thrombin was also enhanced ~1.7-fold. These results may suggest that the size of the S2 pocket is critical for thrombin reaction with DFP. It appears that DFP has preference for a restricted S2 pocket for optimal reaction with thrombin. In the absence of an X-ray crystal structure it is not possible to completely rule out that the L99G mutation did not adversely affect the conformation of the catalytic triad, but a 23.9-fold improved reactivity with PCI, similar inhibition by hirudin, and a normal cofactor function of TM4−6 in the acceleration of the protein C activation rate by this mutant all suggest that most likely the catalytic residues are minimally affected. It is also believed that since residue 99 is not conserved in other members of the serine protease family, it is less likely to play a structural role critical for the folding of these proteins. The reason for the lower k_{cat} values with L99G is likely due to an increase in the size of the S2 pocket impairing stabilization of the transition state of these substrates for efficient catalysis. The higher K_m and k_{cat} values with L99Y and L99T, relative to wild-type thrombin, may reflect nonproductive modes of binding, or a tighter binding of wild-type thrombin to the ground state of the chromogenic substrates which could be detrimental for efficient turnover of these substrates (Fersht, 1985).

All mutants of thrombin reacted with antithrombin at slower rates, but the reactivities of the L99T and L99G thrombins in the absence of heparin were impaired the most. Resolution of the two-step reactions of antithrombin with these mutants in the presence of heparin indicated that the defect in inhibition of mutants occurred in the second step of a reaction mechanism in which an initial reversible complex of thrombin−antithrombin complex is converted to a stable, covalent complex (Olson & Shore, 1982). Antithrombin contains a Gly at the P2 position. Both the structural and mutagenesis data suggest that Thr or Gly at the S2 subsite of thrombin would not be ideal for interaction with a Gly at the P2 position of antithrombin. The X-ray crystal structure of thrombin inhibited by D-Phe-Pro-Arg chloromethyl ketone suggests that the S2 pocket of thrombin is very hydrophobic and ideal for the binding of medium-sized nonpolar residues like Pro (Stubbs & Bode, 1993). A previous mutagenesis study with several P2 mutants of α1-antichymotrypsin demonstrated that the slowest thrombin-reacting serpin variant contained a Thr at the P2 position, suggesting that thrombin will not tolerate a hydrophilic S2−P2 interaction (Djie et al., 1996). Antithrombin, however, inhibited L99T thrombin ~2-fold better than L99G, suggesting that the larger size of Thr fills some of the free space in the S2 pocket of the enzyme, therefore slightly improving its reactivity with antithrombin. These results suggest that an optimal size hydrophobic S2 pocket is necessary for effective thrombin−antithrombin reaction. Antithrombin also inhibited L99Y thrombin ~14-fold slower than wild-type thrombin. This result was surprising since the small size of the P2 Gly of antithrombin would have been expected to be favored by Tyr in this pocket. It is noteworthy, however, that factor Xa (Tyr99), which prefers a Gly at the P2 position of substrates, reacts with antithrombin at an even slower rate than thrombin, suggesting that the conformation of the reactive site loop of antithrombin may not be ideal for recognition by either factor Xa or L99Y thrombin (Huntington et al., 1996).

Scheme 2



In the presence of heparin, all mutants of thrombin were inhibited by antithrombin at moderately high rates and the interesting observation was that the SI values of antithrombin inhibition were altered in the mutant thrombins. The SI of 1.6 for the wild-type was elevated to 4.6 for L99Y and was decreased to 1.2 for L99T and 1.1 for L99G thrombins. These results are best described in the context of a branched pathway, suicide substrate inhibition mechanism proposed for antithrombin and other serpins (Olson, 1985; Fish & Björk, 1979). In this mechanism (Scheme 2), an initial enzyme inhibitor encounter complex (EI) is converted into a tetrahedral or an acyl intermediate complex (EI′) with the rate constant k_3, similar to normal reaction of enzymes with their true substrates (Olson et al., 1995; Olson, 1985; Fish & Björk, 1979). The intermediate (EI′) may continue along the substrate pathway with rate constant k_4 to yield a reactive site cleaved serpin (I*) and free enzyme (E), or it may undergo a conformational change to insert the reactive site loop into β-sheet A with the rate constant k_5, trapping the enzyme in a stable, covalent complex (E−I†) (Potempa et al., 1994). In this branched pathway model of the serpin−enzyme reaction mechanism, the intermediate EI′ is considered a common species to both substrate and inhibitory pathways. Steps leading up to formation of EI′ are typically rate-limiting, so that values of k_4 and k_5, which determine the partitioning of EI′, do not affect the overall rate of reaction (Gettins et al., 1996). Resolution of the two-step L99T and L99G thrombin−antithrombin reactions in the presence of heparin revealed that the equilibrium dissociation constants, K_D, were not impaired by the mutations, but that the rate constant, k_3, leading to the stabilization of the common intermediate, EI′, was impaired in both reactions. In the context of the branched pathway inhibition mechanism, the inhibition kinetic data (Table 2) would suggest that the impairment in the rate constant k_3 in L99T and L99G thrombins is reflected in a 41.8- and 64.5-fold decrease in the inhibitory pathway rate constant, k_5, and a 68% and 75% decrease (Figure 2) in the substrate pathway rate constant, k_4, for these mutants, respectively. Although the slow-binding kinetic approach was not successful for such kinetic studies with L99T and L99G thrombins in the absence of heparin, nevertheless, the impairment in the rate constant, k_3, is the most likely event since it is known that the primary effect of heparin in acceleration of inhibition is the lowering of the K_D for the initial encounter complex formation ($k_2/k_1 = K_D$) with minimal or no role on the second step of the reaction (Olson & Shore, 1982). Consistent with this hypothesis, resolution of the reaction steps for L99Y thrombin in the absence of heparin (Scheme 1) suggests that the second step of the reaction is also impaired by this mutation. The kinetic steps for L99Y thrombin in the presence of heparin were not resolved, but since the SI × k_{assn} value is minimally affected, the overall effect of the L99Y mutation appears only to be the alteration of the partitioning of EI′ at the branch point in favor of enhancing the substrate pathway rate constant, k_4.

The observation that PCI inhibited L99T thrombin at a similar or faster rate than wild-type thrombin suggests that increase in the polarity of the S2 specificity pocket is not detrimental for the PCI−thrombin reaction. PCI inhibited the L99G mutant 23.9-fold faster than thrombin, suggesting that Phe at P2 position of PCI imposes space limitation in fitting into the S2 pocket of thrombin. This hypothesis is consistent with the lower reactivity of L99Y thrombin (Table 3) and factor Xa (Tyr99) with PCI (Pratt & Church, 1993). A factor Xa mutant with a Thr at the S2 position (Rezaie, 1996a) also reacts with PCI with a ~3-fold higher rate than wild-type factor Xa (data not shown). Furthermore, a mutant of factor Xa with a Gly at the S2 position reacted with PCI with a rate more than 20-fold higher than wild-type factor Xa.[3] All of these results indicate that the polarity of the S2 specificity pocket of thrombin or factor Xa may not play a role in determination of the P2 specificity with the serpin, PCI. It appears that only the size of this pocket is critical for the reactions of these proteases with PCI. This finding has important implications for the mechanism by which PCI can inhibit APC. APC is remarkably resistant to inhibition by the plasma inhibitors and, therefore, has a long circulating half-life of 15 min (Heeb et al., 1989; Heeb & Griffin, 1988). It is believed that APC, by having a hydrophilic Thr at position 99, creates an open and hydrophilic S2 specificity pocket, thereby restricting APC from inhibition by the plasma inhibitors, nearly all of which contain small and/or hydrophobic residues at the P2 positions. The exception, however, is PCI which contains a Phe at P2 position yet it inhibits APC at a moderately high rate, so the serpin is called PCI (Pratt & Church, 1993). The results of this study indicate that the polarity of the S2 specificity pocket of these coagulation proteases may not be critical for their reactions with PCI, rather only the size of the S2 pockets dictate specificity with this inhibitor providing a possible explanation for ability of APC to specifically react with PCI. These results are also consistent with previous mutagenesis studies which indicated that substitution of Phe with smaller Pro, Ala, or Gly improved the reactivities of the PCI mutants with thrombin (Cooper & Church, 1995; Phillips et al., 1994).

In a previous study it was demonstrated that TM accelerates PCI inhibition of thrombin more than 100-fold (Rezaie et al., 1995). The observation that L99G reacted with PCI 23.9-fold faster than wild-type thrombin in the absence of TM but at similar rates in the presence of TM suggests that Leu99 is involved in the cofactor function of TM for acceleration of thrombin inhibition by PCI. It has been demonstrated that TM changes the specificity of thrombin from a coagulant to an anticoagulant enzyme by binding to anion binding exosite 1 of thrombin and altering the conformation of the active site pocket of enzyme. TM-induced conformational changes appear to alleviate repulsive interactions between the acidic Glu192 of thrombin and the acidic P3 and/or P3′ Asp residues of protein C (Rezaie & Esmon, 1992; Le Bonniec & Esmon, 1991). The results of this study now suggest that in addition to Glu192, the TM-induced conformational change also involves Leu99 which overcomes the steric inhibitory interaction of this residue with the bulky P2 Phe residue of PCI, analogous to Glu192 accommodating Asp residues at the P3 and P3′ sites of protein C. The observation that TM does not accelerate the PCI inhibition of L99Y and L99T thrombins to the same extent as wild-type thrombin may be understood in terms of the lack of ability of TM to easily reorient a bulkier residue (Tyr) or a hydrophilic residue (Thr) in the restricted and hydrophobic active site pocket of thrombin.

The observation that Leu99 mutations reduced the GDPC activation rates by all mutants to less than 1% of wild-type thrombin suggests that both the size and the polarity of the S2 specificity pocket of thrombin are also critical for protein C activation. The improved activation rates in the presence of TM4−6 supports the hypothesis that TM modulation of the S2 specificity pocket of thrombin involves Leu99. In contrast to the K_m value of 2.9 μM for GDPC activation by the wild-type thrombin−TM4−6 complex, as determined in this study, no K_m determination was possible for any of the mutants since the reaction rates remained linear for concentrations up to 20 μM GDPC. Due to higher K_m values no detailed kinetic analysis was possible to determine whether the k_{cat} values were also impaired in mutant thrombins. Relative to wild-type thrombin, the Leu99 mutation reduced fibrinogen clotting activity to 20−25% for L99T or L99G and <1% for L99Y thrombins at fibrinogen concentrations both well below and well above K_m. Only a decrease in the k_{cat} of fibrinogen cleavage by thrombin could explain the impairment in the rate of fibrinogen cleavage at both low and high concentrations of substrate. In the crystal structure of thrombin in complex with an analogue of fibrinopeptide A, the S2 pocket is occupied with two hydrophobic residues Val15 and Leu9 at the P2 and P8 positions of the peptide, respectively (Stubbs et al., 1992). These residues fit into a hydrophobic pocket created by the side chains of Trp215, Leu99, His57, Tyr60a, and Trp60d (Stubbs & Bode, 1993; Stubbs et al., 1992). The side chain of Tyr in L99Y thrombin is too bulky to fit into this pocket, explaining the dramatic decrease observed in fibrinogen clotting activity of this mutant. The impaired clotting activities of L99T and L99G thrombins further suggest that both the size and the polarity of the S2 specificity pocket of thrombin are critical for the P2 recognition of fibrinogen.

[3] A. R. Rezaie, unpublished results.

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