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Reactivities of the S2 and S3 subsite residues of thrombin with the native and heparin-induced conformers of antithrombin

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Abstract

A pentasaccharide (PS) fragment of heparin capable of activating antithrombin (AT) markedly accelerates the inhibition of factor Xa by AT, but has insignificant effect on inhibition of thrombin. For inhibition of thrombin, the bridging function of a longer polysaccharide chain is required to accelerate the reaction. To study the basis for the similar reactivity of thrombin with the native or heparin-activated conformers of AT, several residues surrounding the active site pocket of thrombin were targeted for mutagenesis study. Leu99 and Glu192, the variant residues influencing the S2 and S3 subsite specificity of thrombin were replaced with Tyr and Gln. The Tyr60a, Pro60b, Pro60c, and Trp60d residues forming part of the S2 specificity pocket were deleted from the B-insertion loop of the wild-type and Leu99/Glu192 → Tyr/Gln thrombins. Kinetic studies indicated that the reactivities of all mutants with AT were moderately or severely impaired. Although heparin largely corrected the defect in reactivities, it also markedly elevated the stoichiometries of inhibition with the mutants. Interestingly, PS also accelerated AT inhibition of the mutants 5–68-fold, suggesting that the mutants are able to discriminate between the native and activated conformers of AT. Based on these results and the recent crystal structure determination of AT in complex with PS, a model for thrombin–AT interaction is proposed in which the S2 and S3 subsite residues of thrombin are critical for recognition of the P2 and P3 residues of AT in the native conformation. In the activated conformation, other residues are made accessible for interaction with the protease, and the similar reactivity of thrombin with the native and heparin-activated conformers of AT may be coincidental. The results further suggest that the S2 and S3 subsite residues are crucial in controlling the partitioning of the thrombin–AT intermediate into the alternative inhibitory or substrate pathways of the reaction.

Keywords: antithrombin; heparin; inhibition; pentasaccharide; specificity; thrombin

Factor Xa and thrombin are trypsin-like serine proteases of the coagulation cascade that react slowly with antithrombin in the absence of heparin, but rapidly in its presence (Damus et al., 1973; Olson & Shore, 1982). Several studies have indicated that heparin accelerates antithrombin inhibition of thrombin and factor Xa by different mechanisms (Choay et al., 1989; Olson et al., 1992). In factor Xa inhibition, a heparin-induced conformational change in the reactive site loop of antithrombin is required for acceleration of the reaction (Choay et al., 1989; Olson et al., 1992). In thrombin inhibition, however, the activation of antithrombin contributes little to inhibition, but the bridging function of heparin is required for acceleration of the reaction (Danielsson et al., 1986; Olson et al., 1992). These distinct inhibition mechanisms are supported by the observation that a unique pentasaccharide fragment of heparin that can bind and activate antithrombin, but because it is not long enough to bridge the inhibitor and enzyme, specifically promotes factor Xa, but not thrombin inhibition (Choay et al., 1989; Olson et al., 1992). For thrombin inhibition, it has been shown that heparin chains containing the pentasaccharide plus at least 13 additional saccharides are required to accelerate the reaction (Lane et al., 1984; Danielsson et al., 1986).

The molecular basis for differences in the reactivity of thrombin and factor Xa with heparin-induced conformer of antithrombin is not known, but comparisons of the crystal structures indicate that active site pocket differences may differentially influence the re-

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Abbreviations: AT, antithrombin; PS, pentasaccharide; SI, stoichiometry of inhibition; thrombin L99Y, thrombin mutant in which Leu99 (in chymotrypsin numbering system of Bode et al. (1989) is substituted with Tyr; E192Q, thrombin mutant in which Glu192 substituted with Gln; L99Y/E192Q, thrombin mutant in which Leu99 and Glu192 are substituted with Tyr and Gln; des-YPPW, thrombin mutant in which is Tyr, Pro, Pro, Trp are deleted from the B insertion loop; des-YPPW/L99Y/E192Q, des-YPPW mutant of thrombin in which Leu99 and Glu192 are substituted with Tyr and Gln; PPACK, Pro-Phe-Arg-chloromethylketone; BSA, bovine serum albumin.

activity of these proteases with antithrombin (Bode et al., 1989; Padmanabhan et al., 1993). The active site of thrombin is occluded by the B and C insertion loops, which impede docking of macromolecular substrates and inhibitors to the active site pocket. These insertion loops are unique to thrombin (Bode et al., 1989). In the crystal structure of thrombin, Tyr60a, Pro60b, Pro60c, and Trp60d of the B-insertion loop form a lid over the S2 specificity pocket of thrombin (Bode et al., 1989; Stubbs & Bode, 1993). There are also several variant residues in the extending binding pockets of thrombin and factor Xa that can influence specificity of these enzymes in their reactions with antithrombin (Bode et al., 1989; Padmanabhan et al., 1993). At the S2 subsite,[1] residue 99 is a variant residue that is shown to be critical for determining the P2 binding specificity of factor Xa and thrombin in their reactions with antithrombin (Rezaie, 1996a, 1997). Residue 192, at the base of the active site pocket, is another variant residue that is known to influence the S3 specificity of thrombin, factor Xa, and other coagulation proteases (Le Bonniec & Esmon, 1991; Rezaie & Esmon, 1993; Neuenschwander & Morrissey, 1995; Rezaie & Esmon, 1995). The critical role of Glu192 in restricting the reactivity of thrombin with the serpin, α_1-antitrypsin, and the Kunitz inhibitors, bovine pancreatic trypsin inhibitor and tissue factor pathway inhibitor, is well studied (Guinto et al., 1994; Le Bonniec et al., 1995). However, the role of this residue in the reaction with antithrombin is not studied in detail.

To investigate the contribution of each one of these different structural elements to the specificity of thrombin reaction with the native and heparin-induced conformers of antithrombin, several mutants of thrombin were prepared in which these residues were either switched to those of factor Xa specificity or deleted to resemble the factor Xa structure. The mutants are: the Leu99 → Tyr substitution (L99Y), the Glu192 → Gln substitution (E192Q), the Leu99/Glu192 → Tyr/Gln substitutions (L99Y/E192Q), the YPPW deletion (des-YPPW), and finally the YPPW deletion together with the Leu99/Glu192 → Tyr/Gln substitutions (des-YPPW/L99Y/E192Q). The reactivities of these S2 and S3 subsite mutant enzymes with antithrombin in the absence or presence of heparin or the pentasaccharide were examined. In general, the mutants reacted with antithrombin poorer than wild-type thrombin in the absence of either co-factor. Heparin, however, accelerated the reaction rates to nearly normal levels, but the stoichiometry of inhibition (SI) in the presence of heparin was elevated with the mutants, suggesting that the mutations contribute to better recognition of antithrombin in the substrate pathway of the reactions. The template effect of heparin was not affected by the mutations after correction for SI. In contrast to an insignificant effect in the acceleration of thrombin inhibition, the pentasaccharide markedly accelerated the inhibition of the mutants by antithrombin, suggesting that the native and activated conformations of antithrombin are recognized differently by the mutants. Based on these results and the recent crystal structure determination of antithrombin (Skinner et al., 1997) and antithrombin–pentasaccharide complex (Carrell et al., 1997), a model for the thrombin–antithrombin reaction is proposed in which the P2 and P3 residues of antithrombin are more important for recognition of the native conformation but less important for recognition of the activated conformation by thrombin. In the activated conformation, it appears that residues other than P2 and P3 are made accessible for interaction with thrombin. In contrast to factor Xa, however, both the native and activated conformers of antithrombin are sub-optimal for recognition by thrombin and that the similar reactivity of thrombin with the two conformers of antithrombin may be coincidental. The effect of the mutations on the SI values further suggests that the S2 and S3 subsites of thrombin are crucial in controlling the partitioning of the reaction intermediate into the alternative substrate or inhibitory pathways of the reaction in the context of a branched pathway, suicide substrate inhibition mechanism proposed for most serpins (Gettins et al., 1996).

[1]Nomenclature of Schechter and Berger (Schechter & 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 as P1′, P2′, etc. The corresponding sites on the enzyme where substrate residues interact are designed S1, S2,... and S1′, S2′,..., respectively.

Results

The prethrombin-1 forms of the mutant proteins were activated to thrombin and purified to homogeneity as described in Materials and methods. Recombinant wild-type, E192Q, and des-YPPW thrombins all migrated with an apparent molecular mass of ~37 kDa on SDS-PAGE gels. Thrombin L99Y migrated as two bands with equal intensities at ~37 and ~38 kDa, and thrombin des-YPPW/L99Y/E192Q migrated primarily a 38 kDa band, suggesting that the L99Y mutation is associated with differential glycosylation rates in the mutant thrombins. With thrombin E192Q, L99Y/E192Q, and des-YPPW/L99Y/E192Q, two additional bands corresponding to ~27 and ~15 kDa were observed. These bands are most likely the autolytic fragments of thrombin referred to as γ-thrombin. In γ-thrombin, cleavages after Arg75, and/or Arg77a, and Lys149e by a slow autolytic process, or more efficiently by trypsin (γ_T-thrombin), result in the fragmentation of thrombin into three polypeptides (Chang et al., 1979). It appears that substitution of Glu192 with Gln, the same residue at the identical site of trypsin, confers a trypsin-like specificity upon thrombin that accelerates the autolytic process. This was observed with all Glu192 → Gln substitution mutants of thrombin. The highest ratio of γ-thrombin to α-thrombin was observed with the L99Y/E192Q mutant.

Chromogenic substrate specificity of thrombin mutants

To examine the influence of mutations 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). All mutants hydrolyzed SpTH and SpPCa better than wild-type thrombin at saturating concentrations of substrates. As shown in Table 1, the k_{cat} constants for hydrolysis of all these chromogenic substrates were increased relative to thrombin. However, the increases in the k_{cat} constants were accompanied by an increase in the K_m values, resulting in an overall lower catalytic specificity (k_{cat}/K_m). L99Y thrombin cleaved SpFXa (P2 Gly) with the k_{cat} and K_m values poorer than wild-type thrombin, but when Glu192 in this mutant was also replaced with Gln, the resulting L99Y/E192Q mutant cleaved SpFXa with a higher k_{cat} constant, but the K_m still remained higher than that of wild-type thrombin. The higher K_m values in this mutant appears to be the result of the L99Y substitution, because a previous study with the single E192Q mutant demonstrated that the K_m for this substrate remained un-

Table 1. *Steady-state kinetics of chromogenic substrate hydrolysis by thrombin, thrombin mutants, and factor Xa*[a]

	SpTH			SpPCa			SpFXa			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[a]	4.2	17.1	4.1	4.9	21.4	4.4	51.0	6.2	0.1	7.6	31.0	4.1
L99Y[a]	54.0	31.8	0.6	35.7	53.4	1.5	104.7	1.8	0.02	24.0	44.1	1.8
L99Y/E192Q	379.4	18.7	0.05	257.9	43.0	0.17	220.8	13.1	0.06	327.8	22.9	0.07
des-YPPW	24.4	40.3	1.65	79.5	29.7	0.37	535.3	6.7	0.01	463.4	24.2	0.05
des-YPPW/L99Y/E192Q	473.5	18.4	0.04	252.6	24.7	0.10	558.3	17.7	0.03	633.1	6.7	0.01
Factor Xa	524.1	25.5	0.05	165.6	8.9	0.05	145.3	47.9	0.33	106.5	0.9	0.008

[a]The kinetic constants K_m (μM), k_{cat} (s^{-1}), and k_{cat}/K_m ($\mu M^{-1} s^{-1}$) values for each chromogenic substrate were determined as described under Materials and methods.

[b]Except for SpFXa, the kinetic values for the wild-type and L99Y thrombins are derived from a previous publication (Rezaie, 1997). All values are the average of at least three independent measurements with a ±SD values of 7% or less for the K_m determinations and 10% or less for the k_{cat} determinations.

changed, but the k_{cat} was improved ~2.5-fold (Le Bonniec & Esmon, 1991). All other mutants cleaved SpFXa with a similar or higher k_{cat}, but with an increased K_m values (Table 1).

Reaction with antithrombin

The second-order association rate constants (k_2) for antithrombin inhibition of thrombin and the mutants in the presence and absence of heparin or the pentasaccharide are shown in Table 2. Antithrombin inhibited thrombin with $k_2 = 1.1 \pm 0.1 \times 10^4\ M^{-1} s^{-1}$. The rates of inhibition of L99Y, E192Q, and L99Y/E192Q thrombins by antithrombin were 14.1-, 5.2-, and 7.3-fold slower than thrombin, respectively. The reactivities of des-YPPW and des-YPPW/L99Y/E192Q with antithrombin were severely impaired, as antithrombin inhibited these mutants 1,222.2- and 440.0-fold, respectively, slower than wild-type thrombin (Table 2). Heparin accelerated antithrombin inhibition of all thrombin mutants by a template mechanism, as evidenced by the existence of an optimal concentration of heparin for acceleration of the reactions (Fig. 1). The optimal concentration of heparin ranged from 0.25–0.5 U/mL heparin for all thrombin derivatives under the experimental conditions described under Materials and methods. Heparin corrected most of the defect in antithrombin reactivities observed with these mutants. The inhibition promoting effect of heparin was 2,273-fold for wild-type, 5,256-fold for L99Y, 2,095-fold for E192Q, 2,000-fold for L99Y/E192Q, 70,000-fold for des-YPPW, and 144,000-fold for des-YPPW/L99Y/E192Q thrombins (Table 3).

To determine whether such elevated heparin rate enhancement of des-YPPW and des-YPPW/L99Y/E192Q inhibition is due to the bridging function of heparin or to whether the mutations have altered the specificity of thrombin so that the mutants now recognize the active conformer of the serpin, the inhibition reactions were carried out in the presence of the pentasaccharide. Interestingly, as shown in Table 2, the pentasaccharide accelerated the inhibition of the mutants better than wild-type thrombin. The rate enhancement of the inhibition of des-YPPW/L99Y/E192Q thrombin was the highest, a 68.0-fold increase in the inhibition rate constant ($k_2 = 1.7 \pm 0.4 \times 10^3\ M^{-1} s^{-1}$). The pentasaccharide accelerated the inhibition of wild-type thrombin by antithrombin ~1.7-fold. The acceleration of inhibition by the pentasaccharide was improved to 5.0-fold for L99Y, 10.5-fold for E192Q, 10.7-fold for L99Y/E192Q, and 17.8-fold for des-YPPW thrombins (Table 3).

It has been demonstrated that the active conformer of antithrombin, whether it is activated by heparin or the pentasaccharide, contributes similarly to the acceleration of protease inhibition (Olson et al., 1992). It should, therefore, be possible to estimate the extent that the bridging function of heparin contributes to the rate enhancements observed with the thrombin mutants. This can be done by dividing the inhibition rates determined in the presence of

Table 2. *The second-order association rate constants for antithrombin inhibition of recombinant wild-type and mutant thrombins in the presence and absence of heparin or pentasaccharide*[a]

	k_2 ($M^{-1} s^{-1}$) without heparin	k_2 ($M^{-1} s^{-1}$) with PS	k_2 ($M^{-1} s^{-1}$) with heparin
Thrombin	$1.1 \pm 0.1 \times 10^4$	$1.9 \pm 0.2 \times 10^4$	$2.5 \pm 0.1 \times 10^7$
L99Y	$7.8 \pm 0.2 \times 10^2$	$3.9 \pm 0.5 \times 10^3$	$4.1 \pm 0.2 \times 10^6$
E192Q	$2.1 \pm 0.1 \times 10^3$	$2.2 \pm 0.2 \times 10^4$	$4.4 \pm 0.2 \times 10^6$
L99Y/E192Q	$1.5 \pm 0.1 \times 10^3$	$1.6 \pm 0.1 \times 10^4$	$3.0 \pm 0.2 \times 10^6$
des-YPPW	$0.9 \pm 0.1 \times 10^1$	$1.6 \pm 0.2 \times 10^2$	$6.3 \pm 0.3 \times 10^5$
des-YPPW/L99Y/E192Q	$2.5 \pm 0.1 \times 10^1$	$1.7 \pm 0.4 \times 10^3$	$3.6 \pm 0.2 \times 10^6$

[a]The second-order association rate constants (k_2) in the absence and presence of heparin or the pentasaccharide were determined from the inhibitions assays described under Materials and methods.

Table 3. *Heparin or pentasaccharide acceleration of antithrombin inhibition of the recombinant wild-type and mutant thrombins*

	Acceleration[a] (-fold) PS	Acceleration (-fold) heparin	Template effect[b] (-fold)	SI-corrected[c] template effect
Thrombin	1.7	2,273	1,337	2,006
L99Y	5.0	5,256	1,051	4,835
E192Q	10.5	2,095	200	1,940
L99Y/E192Q	10.7	2,000	187	2,637
des-YPPW	17.8	70,000	3,933	5,900
des-YPPW/L99Y/E192Q	68.0	144,000	2,118	6,354

[a]The data are shown as the -fold acceleration of the inhibition rate due to the pentasaccharide or heparin. The acceleration due to each cofactor was calculated by dividing the k_2 values of inhibition in the presence of the cofactor (Table 2, columns 3 and 4) by the same values in the absence of the cofactor (Table 2, column 2).

[b]Calculated as the ratio of data presented in column 3 (heparin) to column 2 (PS).

[c]The values of column 4 are multiplied by the SI values (Table 4).

heparin by the rates determined in the presence of the pentasaccharide. The ratios of these rate constants are shown in Table 3. Analysis of these results indicate that the bridging mechanism of heparin contributes 1,337-fold for acceleration of the reaction with wild-type thrombin. The template effect of heparin was found to be 1,051-fold inhibition rate enhancement for L99Y, 200-fold for E192Q, 187-fold for L99Y/E192Q, 3,933-fold for des-YPPW, and 2,118-fold for des-YPPW/L99Y/E192Q thrombins. The observation that the template function of heparin was impaired with E192Q and L99Y/E192Q suggested that the mutants may recognize antithrombin as a substrate. Several studies in the past have indicated that heparin increases the SI values of complex formation from ~1 to ~1.5 for both thrombin and factor Xa (Olson et al., 1992). To determine whether the mutations influenced the substrate pathway of the reactions, the SI values of complex formation were determined for the mutants in the absence and presence of heparin or the pentasaccharide. The results indicated that antithrombin, both in the absence and presence of pentasaccharide, inhibited all thrombin derivatives with a 1:1 stoichiometry, consistent with results observed by others for wild-type thrombin (data not shown). In the presence of heparin, however, the SI values for all of these mutants were increased by varying degrees (Fig. 2). The highest SI value was observed with E192Q and L99Y/E192Q offering an explanation for the lower apparent heparin template effect observed for these mutants. As expected, the template effects are similar after the rate constants are corrected for SI. Because the mutants increased the SI values by different extents, the effective inhibition rate constant, k_2(SI), was calculated for each mutant for an accurate comparisons of the rate values (Table 4). For an accurate assessment of the extent of the heparin acceleration effect, the effective inhibition rate constants determined in the presence of heparin were divided by the inhibition rate constants determined in the absence of heparin [$k_2(SI)_{hep}/k_2$], and the ratios are presented in the last column of Table 4. Similarly, the SI-corrected template effects are calculated and listed as a separate column in Table 3 (last column).

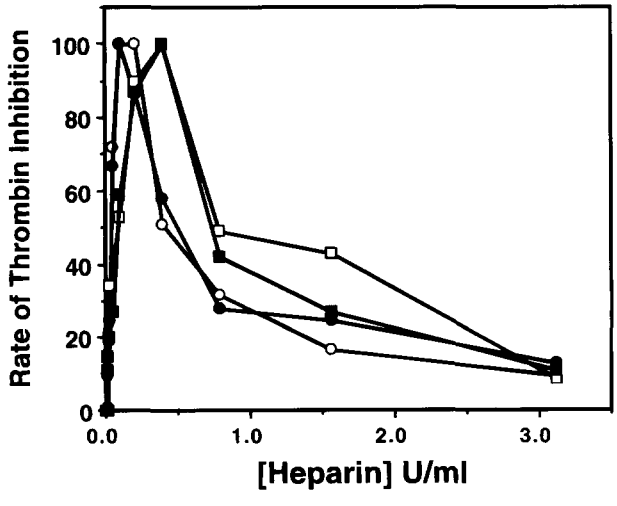

Fig. 1. Heparin concentration dependence of wild-type, E192Q, L99Y/E192Q, and des-YPPW/L99Y/E192Q thrombin inhibition by antithrombin. The antithrombin inhibition of wild-type (open circles), E192Q (filled circles), L99Y/E192Q (open squares), and des-YPPW/L99Y/E192Q (filled squares) thrombins were monitored as a function of different heparin concentrations as described under Materials and methods. The inhibition rates were normalized to a maximum rate of 100. The actual inhibition rate constants are given in Tables 2 and 4.

To directly monitor the extent of complex formation between the thrombin mutants and antithrombin, SDS-PAGE analysis was performed following incubation of an equimolar amount of each thrombin derivative with antithrombin in the presence or absence of heparin. As shown in Figure 3A, L99Y, E192Q, and L99Y/E192Q thrombins (lanes 3–5) all formed high molecular weight complexes with antithrombin in the absence of heparin. In the presence of heparin, however, the mutants cleaved antithrombin, as evidenced by lack of high molecular weight complexes and a decrease in the mobility of antithrombin on SDS-PAGE under non-reducing conditions (lanes 7 and 8), suggesting that the inhibitor is cleaved. Others have also reported that the cleaved antithrombin migrates slightly slower than intact antithrombin under non-reducing conditions (Olson, 1985). A similar SDS-PAGE analysis in the presence of pentasaccharide indicated that the mutants formed high molecular weight complexes with antithrombin, with no indication of the cleavage of the inhibitor (data not shown). This suggests that similar to thrombin, inactivation of these mu-

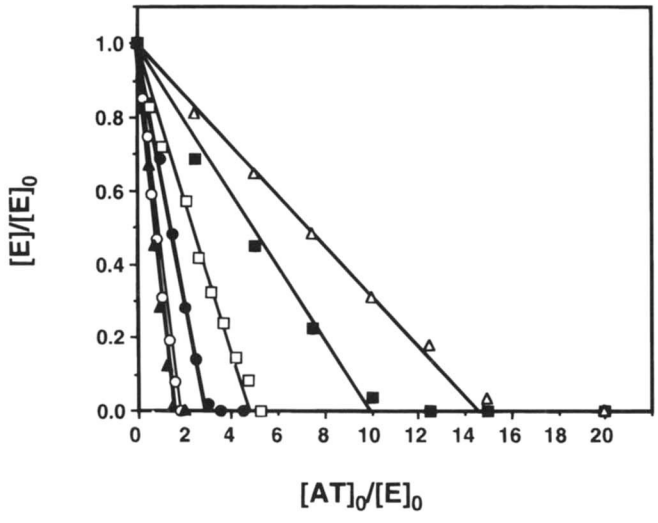

Fig. 2. Determination of the stoichiometries of inhibition for the recombinant wild-type and mutant thrombins in the presence of heparin. Titration of fixed amount of each thrombin derivative (100 nM) with increasing concentration of antithrombin was monitored from the residual amidolytic activities as described under Experimental procedures. The symbols are: (open circle) thrombin, (filled triangles) des-YPPW, (filled circles) des-YPPW/L99Y/E192Q, (open squares) L99Y, (filled squares) E192Q, (open triangles) L99Y/E192Q. The solid lines are linear regression fits of inhibition data.

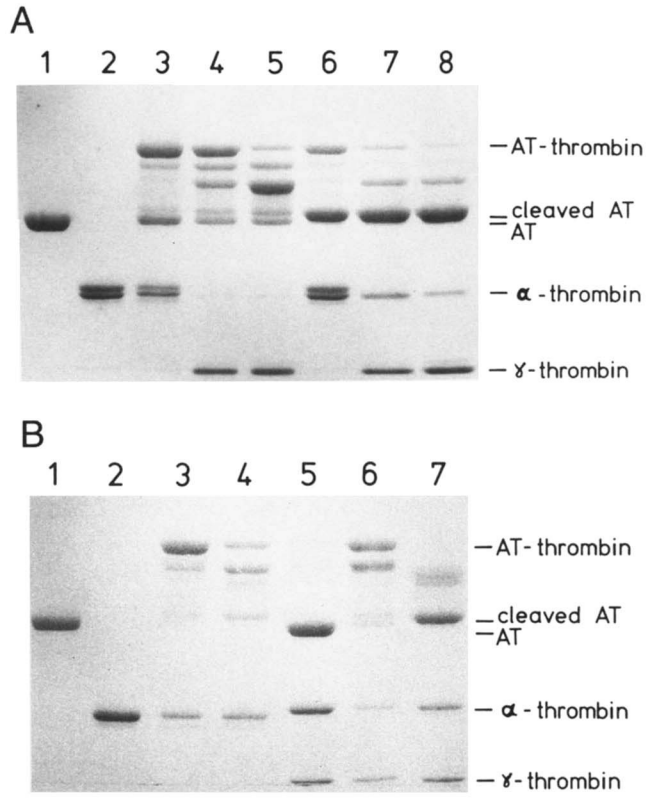

Fig. 3. SDS-PAGE analysis of the stable thrombin–antithrombin complexes. **A:** Anti-thrombin (2.5 μM) was incubated with equimolar concentrations of thrombin for 20 min in the absence of heparin or 2–3 min in the presence of heparin in 50 μL reactions in TBS at room temperature. The reactions were stopped by addition of PPACK to a final concentration of 200 μM, and 10 μL of five times non-reducing sample buffer was added and the samples were boiled for 5 min. Twenty microliters of the inhibition reactions were loaded on 10% gel. The stable protease–AT complexes migrated as the highest molecular weight band, followed by degraded complexes. Under non-reducing conditions, the reactive site cleaved antithrombin migrated slightly slower than native AT. Lane 1, native AT; lane 2, thrombin L99Y; lanes 3–5, L99Y, E192Q, and L99Y/E192Q incubated with antithrombin in the absence of heparin, respectively; lanes 6–8, the same thrombin mutants incubated with antithrombin in the presence of heparin, respectively. All mutants containing an E192Q substitution contained the autolytic product γ-thrombin as two bands of ~27 and ~15 kDa. The ~15 kDa band migrated at the dye front and it was purposefully let run out of the gel to separate the closely migrating cleaved and uncleaved forms of AT. **B:** The experimental conditions are the same as those described above. Lane 1, native AT; lane 2, recombinant wild type thrombin; lanes 3 and 4, recombinant wild-type thrombin incubated with antithrombin in the absence and presence of heparin, respectively. Lane 5, des-YPPW/L99Y/E192Q incubated with antithrombin in the absence of heparin or the pentasaccharide; lanes 6 and 7, the same mutant incubated in the presence of the pentasaccharide and heparin, respectively.

tants is due to the formation of 1:1 stoichiometric complexes. In Figure 3B, a similar SDS-PAGE analysis is shown for the recombinant wild-type and des-YPPW/L99Y/E192Q thrombins. In the absence of heparin, thrombin formed high molecular weight complexes with antithrombin with a minimum cleavage of the inhibitor (lane 3). In the presence of heparin, however, and consistent with the increased SI value, the amount of high molecular weight thrombin–antithrombin complexes were reduced and the amount of cleaved antithrombin was increased (lane 4). With des-YPPW/L99Y/E192Q in the absence of heparin or pentasaccharide, very little high molecular weight complex was formed and no cleaved antithrombin was observed (lane 5). This is consistent with a lack of reactivity of this mutant with antithrombin (see Table 2 for the inhibition rate constants). Interestingly, in the presence of pentasaccharide this mutant formed high molecular weight complexes with antithrombin rather efficiently (lane 6) supporting the kinetic data shown in Table 1. Similar to other mutants, des-YPPW/L99Y/E192Q also cleaved antithrombin, consistent with the higher SI value observed for this mutant (lane 7).

Table 4. *Stoichiometries of inhibition and the effective inhibition rate constants for the recombinant wild-type and mutant thrombins in the presence of heparin*

	SI[a]	k_2 (SI)[b] ($M^{-1}s^{-1}$)	Acceleration (-fold)[c]
Thrombin	1.5 ± 0.1	3.8×10^7	3,455
L99Y	4.6 ± 0.2	1.9×10^7	24,359
E192Q	9.7 ± 1.0	4.3×10^7	20,476
L99Y/E192Q	14.1 ± 0.7	4.2×10^7	28,000
des-YPPW	1.5 ± 0.1	9.5×10^5	105,556
des-YPPW/L99Y/E192Q	3.0 ± 0.1	1.1×10^7	440,000

[a]The SI values were determined from Figure 2 as described under Materials and methods.

[b]The effective inhibition rate constants were calculated by multiplying the k_2 values in the presence of heparin (Table 2, column 4) by the SI values.

[c]Total heparin acceleration effect after correction for the SI values, calculated as the ratio of data presented in column 3 of Table 4 to data presented in column 2 of Table 2.

The γ-thrombin derivatives of E192Q, L99Y/E192Q, and des-YPPW/L99Y/E192Q appear as ~27 kDa bands in Figure 3. With des-YPPW/L99Y/E192Q in the presence of pentasaccharide, the intensity of the 27 kDa fragment of γ-thrombin was reduced, suggesting that the γ-thrombin derivative of this mutant also recognized the active-conformer of antithrombin to form a high molecular weight complex with the inhibitor (Fig. 3B, lane 6). This indicates that the γ-derivative of this mutant also reacts normally with antithrombin, in a manner similar to γ-thrombin (Chang et al., 1979). With the E192Q and L99Y/E192Q mutants (Fig. 3A), comparison of the ratios of unreacted α- and γ-thrombins in the absence (lanes 4 and 5) and presence (lanes 7 and 8) of heparin appears to suggest that the γ-thrombins of these mutants do not react with antithrombin or they react slowly. Inhibition time-course analysis indicated that antithrombin inactivates all amidolytic activities of these mutants, which suggest that the γ-derivatives of these mutants react with antithrombin, but probably at a slower rate than wild-type thrombin. The observation that the active site concentration of all mutants, as determined with the biotinylated synthetic inhibitor FPR-CK (Materials and methods), was within 90% of that calculated from their absorbance at 280 nm, together with a one to one stoichiometries of inhibition (overnight incubation) further suggest that the γ-derivatives of these mutants can react with antithrombin. The relative antithrombin reaction rates with the isolated γ-derivatives of the thrombin mutants were not studied, but if antithrombin reacts with these derivatives slower than α-forms, then the k_2 values calculated for these mutants may be underestimated. This potential problem will not change the major finding of this study that these mutants discriminate between the native and heparin-induced conformers of antithrombin.

The fibrinogen clotting and protein C activation properties of E192Q (Le Bonniec & Esmon, 1991), PPW loop deletion (Le Bonniec et al., 1993), and L99Y (Rezaie, 1997) mutants of thrombin were studied previously, and not further studied here.

Discussion

Heparin acceleration of factor Xa inhibition by antithrombin is mediated by an activation mechanism involving a conformational change in the reactive site loop of the serpin, but in thrombin inhibition a trimolecular complex formation by the bridging mechanism of heparin is required for acceleration of the reaction (Danielsson et al., 1986; Olson et al., 1992). In this study, the contribution of the S2 and the S3 subsite residues, Leu99, Glu192, and the B-insertion loop to specificity of the thrombin reaction with antithrombin was investigated. Of particular interest was to evaluate whether these residues play a role in the inability of thrombin to differentiate the native and the activated conformations of antithrombin. The experimental strategy was to generate several mutants of thrombin in which each one of these residues either alone or in combination were switched to those of the factor Xa specificity, and then examine the reactivity of each mutant with antithrombin in the absence and presence of the pentasaccharide fragment of heparin, which is known to accelerate the inhibition of factor Xa, but not that of thrombin. Similar to heparin, the pentasaccharide binds and activates antithrombin to accelerate the inhibition of factor Xa, but it is not long enough to bridge the enzyme-inhibitor for an approximation effect, which is necessary for the acceleration of thrombin inhibition (Olson et al., 1992).

As with any mutagenesis study, it was first necessary to ascertain that the mutations did not adversely affect the folding or the function of the mutant proteins. The kinetics of hydrolysis of several chromogenic substrates by the thrombin mutants suggest that none of the mutations caused any deleterious conformational change that would impair the charge stabilizing system or the reactivity of the catalytic triad, because at the saturating concentrations of the chromogenic substrates, the mutant enzymes generally exhibited higher or nearly normal k_{cat} values (Table 1). In a previous study it was demonstrated that exchange of residue 99 between factor Xa and activated protein C essentially switched the specificity of these enzymes with respect to hydrolysis of their specific chromogenic substrates (Rezaie, 1996a). In contrast to factor Xa and activated protein C, however, the exchange of Leu99 and/or Glu192 of thrombin with factor Xa specific residues, Tyr and/or Gln, did not switch the chromogenic substrate specificity of thrombin. The mutant enzymes hydrolyzed Spectrozyme FXa with specificity constants that were lower than wild-type thrombin, suggesting that the mutations did not result in proteases with factor Xa-like specificity.

Comparisons of the inhibition rate constants indicate that the mutations impaired the reactivities of the mutant enzymes with antithrombin. The pentasaccharide however, accelerated inhibition of all mutants by antithrombin significantly better than that of wild-type thrombin, which resulted in a partial or complete correction of the defect in serpin reactivity caused by the mutations. This was most apparent with the des-YPPW/L99Y/E192Q mutant where the antithrombin inhibition rate was accelerated 68-fold by the pentasaccharide. None of the thrombin mutants, however, reacted better than wild-type thrombin with the activated antithrombin conformer, as judged from the second-order rate constants. Nevertheless, the thrombin mutants reacted differentially with the two antithrombin conformers similar to what is observed with factor Xa. This follows from the relative values of the second-order rate constants measured in the absence and presence of the pentasaccharide. Insight into the molecular basis for differential reactivities of thrombin mutants with the native and active conformers of antithrombin may be obtained from the recent crystal structure determination of a dimeric form of intact antithrombin (Skinner et al., 1997), and the structures of the pentasaccharide complexes of inactivated and activated antithrombin (Carrell et al., 1997). The structural data support the excellent previous spectral data that the reactive site loop of antithrombin undergoes extensive conformational change upon activation by heparin or the pentasaccharide (Olson et al., 1992). Interestingly, it has been found in the crystal structure that in the absence of the pentasaccharide, the P1 Arg of antithrombin points internally and the reactive site loop conformation is distorted from the canonical form required to fit the active site of protease (Carrell et al., 1997). Activation by pentasaccharide therefore, has been proposed to involve creating a canonical conformation, and presumably also the expulsion of the P1 Arg residue. The pentasaccharide acceleration of antithrombin inhibition of the mutants in the context of these structural data may suggest that P2 and P3 residues play a critical role for thrombin recognizing the native conformation of antithrombin, but probably play a minor role in recognition of the activated conformation. According to this hypothesis, mutations in the S2 and S3 sites would be expected to greatly decrease recognition of the native, but not the activated conformation. In the activated conformation of antithrombin, P1 Arg and/or other newly available residues in the canonical conformation would be expected to partially or completely correct the defect in serpin reactivity caused by the mutations. The observation that the pentasaccharide accelerated antithrombin inhibition of des-YPPW mutant ~10-fold worse than

that of des-YPPW/L99Y/E192Q inhibition could be understood in terms of the latter mutant having factor Xa-specific S2 and S3 subsite residues, which are more optimal than those of thrombin for recognition of the activated conformer of antithrombin. This is consistent with antithrombin having a reactive site loop sequence (from P4 to P1: Ile-Ala-Gly-Arg), which is a poor recognition site for thrombin, but optimal for recognition by factor Xa (Huntington et al., 1996). In native antithrombin, however, the reactive site sequence does not have the optimal conformation for recognition by the active site of factor Xa until heparin or pentasaccharide binds on this site to change the conformation of this sequence. In contrast to factor Xa, however, neither the native nor the activated conformation of antithrombin is optimal for recognition by thrombin, and the similar reactivities of thrombin with two conformers reflect the fortuitous similar reactivity with the P1–P3 residues in the native conformation and with the P1 and/or other newly available reactive site loop residue(s) in the activated conformation.

The other interesting observation of this study is that heparin, but not pentasaccharide, markedly increased the SI values for inhibition of the mutant enzymes. It has been proposed that similar to other serpins, antithrombin inactivates thrombin and factor Xa by a branched pathway, suicide substrate inhibition mechanism in which an initial loose enzyme-inhibitor encounter complex is converted into a tetrahedral or an acyl intermediate complex similar to the normal reaction of enzymes with their true substrates (Fish & Björk, 1979; Olson, 1985; Olson et al., 1995). The intermediate is thought to undergo a conformational change that results in insertion of the reactive site loop into β-sheet A trapping the enzyme in a stable, covalent complex. The intermediate, however, may continue along the substrate pathway and yield a reactive site cleaved serpin, and free enzyme (Potempa et al., 1994; Gettins et al., 1996). In the context of this model, the results of this study indicate that heparin increases the rate of partitioning of the mutant thrombin-inhibitor intermediates into the substrate pathway of the reactions. It has been demonstrated that both heparin and the pentasaccharide similarly increase the SI values ($\sim$1.5) for antithrombin inhibition of factor Xa (Olson et al., 1992). An equimolar 1:1 stoichiometry of inhibition in the presence of pentasaccharide as was observed for all thrombin mutants suggests that unlike factor Xa, the activated conformation of antithrombin does not play a role in recognition of the serpin as a substrate by the wild-type or the mutant thrombins in the presence of heparin. These results are consistent with the finding of Olson et al. (Olson et al., 1992) that a trimolecular complex formation is required for heparin to accelerate the substrate pathway of antithrombin–thrombin reaction. The observation that the YPPW loop deletion from the L99Y/E192Q mutant reduces the SI significantly further suggests that Tyr99 and Gln192 can increase the substrate pathway of the reaction only in context of an intact B-insertion loop. The implication of this observation for the wild-type thrombin–antithrombin reaction is that Leu99 and Glu192 prevent the partitioning of antithrombin into the substrate pathway of the inhibition reaction. Both of these residues therefore, are critical for the inhibitory mechanism of the antithrombin reaction.

In summary, it is shown in this study that the S2 and S3 subsite mutants of thrombin react differentially with the pentasaccharide complexed antithrombin supporting the spectral (Olson et al., 1992) and crystallographic (Carrell et al., 1997; Skinner et al., 1997) data that heparin activation of antithrombin is associated with a change in the conformation of the reactive site loop of the serpin. The results further suggest that the S2 and S3 subsite residues in the extended binding pocket of thrombin play a crucial role in controlling the outcome of thrombin–antithrombin reaction in the alternative substrate or inhibitory pathways of the reactions.

Materials and methods

Mutagenesis, expression, and purification of recombinant proteins

The expression and purification of recombinant wild-type, E192Q, and L99Y, thrombins have been described previously (Rezaie, 1996b, 1997). E192Q, L99Y/E192Q, des-YPPW, and des-YPPW/L99Y/E192Q mutants of thrombin were prepared by the standard polymerase chain reaction (PCR) mutagenesis methods as described (Higuchi et al., 1988). All manipulations were carried out with 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. All mutations were confirmed by DNA sequencing (Sanger et al., 1977). The expression vector containing the prethrombin-1 cDNA fragment or its mutant derivatives were transferred to baby hamster kidney (BHK) cells by Lipofectin (Gibco BRL, Gaithersburg, Maryland) 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 mg/L cell culture supernatant.

Prethrombin-1 activation by the prothrombinase complex and purification on an FPLC Mono S column (Pharmacia, LKB) was described previously (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 E192Q, 17.5 for both L99Y and L99Y/E192Q, 15.3 for des-YPPW and 15.6 for des-YPPW/L99Y/E192Q. 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., Vermont) as the active site probe. The concentration of active enzyme determined by this method agreed within 10% of the values calculated based on absorbance at 280 nm. Bovine antithrombin was prepared as described (Owen, 1975). All proteins were homogeneous as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE).

Spectrozymes TH (SpTH), PCa (SpPCa), and FXa (SpFXa) were purchased from American Diagnostica, Greenwich, Connecticut. The chromogenic substrate S2238 was purchased from Kabi Pharmacia/Chromogenix, Franklin, Ohio. Polybrene was purchased from Sigma. The AT-binding pentasaccharide (N-sulfate-6-O-sulfate-D-glucosamine)α1 $\rightarrow$ 4(D-glucuronic acid)β1 $\rightarrow$ 4(N-sulfate-3,6-di-O-sulfate-D-glucosamine)α1 $\rightarrow$ 4(2-O-sulfate-L-iduronic acid)α1 $\rightarrow$ 4(N-sulfate-3,6,-di-O-sulfate-D-glucosamine) was a generous gift from Dr. Jeffrey Weitz (Hamilton Civic Hospitals Research Centre, Ontario, Canada). Unfractionated heparin

(porcine intestinal mucosa, sodium salt, grade II) was purchased from Sigma.

Kinetic methods

The rate of inhibition of human thrombin and the mutants in the absence or presence of the pentasaccharide and heparin were measured under pseudo-first-order rate conditions by an end point assay method. Thrombin derivatives (2 nM) were incubated with at least a 10-fold molar excess of antithrombin in the absence or presence of 20 μM pentasaccharide at room temperature for up to 20 min in Tris-HCl (pH 7.5), 0.1 M NaCl, I = 0.12 (TBS), buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The volume of the reactions were usually 50 μL in a 96-well polystyrene plate. All experiments were performed in triplicate wells and all experiments contained control wells without added antithrombin. After 5, 10, 15, and 20 min of incubation, 50 μL SpTH was added to a final concentration of 0.4 mM, and the residual enzyme activities were determined by measuring the absorbance at 405 nm with a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, California). The second-order association rate constants were calculated using the equation $k_2 = (-\ln a)/t[I]$ where a is residual protease activity, t is time, and $[I]$ is the concentration of antithrombin. The inhibition of thrombin des-YPPW and des-YPPW/L99Y/E192Q in the absence of pentasaccharide was very slow, so that the maximal antithrombin concentration was increased to 20 μM and the incubation time increased to 60 min. In all assays, less than 10% chromogenic substrate was utilized and at least 50% of enzyme activity was inhibited for calculation of rate constants.

To measure the second-order inhibition rate constants in the presence of heparin, initially the heparin concentrations dependence of the inhibition reactions were determined. In the inhibition assays, 0.25–0.50 nM thrombin was incubated under pseudo-first-order rate conditions with 5–10 nM antithrombin and 0–100 U/mL heparin in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. After a period of time (10–25 s), SpTH in TBS buffer containing 1 mg/mL Polybrene (to block heparin function immediately) was added to give a final concentration of 0.4 mM and the residual thrombin activities were measured as described above. The results indicated that the optimal concentration of heparin to observe the maximal inhibition was 0.25–0.5 U/mL heparin for all thrombin derivatives. With the thrombin mutants in which the SI values of inhibition were elevated, the antithrombin concentrations in the inhibition reactions were at least 10 SI times in excess of the thrombin concentrations. The residual thrombin activities and the second-order association rate (k_2) from at least four time points (10, 15, 20, and 25 s) were estimated as described above.

Determination of inhibition stoichiometry

Except for des-YPPW and des-YPPW/L99Y/E192Q, which reacted with antithrombin very slowly, the SI values were determined for all thrombin derivatives in the absence or presence of 2 U/mL heparin. The SI values were determined by titration of 100 nM thrombin with increasing concentrations of antithrombin corresponding to antithrombin/thrombin molar ratios of 0 to 20, in TBS containing 1 mg/mL BSA and 0.1% PEG 8000. The residual amidolytic activities of thrombin and the mutant enzymes were monitored for up to 24 h at room temperature by the hydrolysis of SpTH 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 inhibition data. The SI values were also determined in the absence and presence of 20 μM pentasaccharide. In this case, 200 nM thrombin or mutant was titrated with increasing concentrations of anti-thrombin corresponding to antithrombin/thrombin molar ratios of 0 to 2. The residual amidolytic enzyme activities were monitored for up to 24 h at room temperature by the hydrolysis of SpTH, and the SI values were determined as described above.

Analysis of thrombin–antithrombin reaction products by gel electrophoresis

Complex formation of thrombin or the mutants with antithrombin was monitored by SDS-PAGE. The reactions were carried out in 50 μL at room temperature with 2.5 μM thrombin derivatives and 2.5 μM antithrombin for 20 min in the absence of heparin and for 2–3 min in the presence of 5 U/mL heparin. The reactions were stopped by addition of Pro-Phe-Arg-chloromethyl ketone (PPACK) to a final concentration of 200 μM and then 10 μL of 5 × non-reducing SDS sample buffer was added and the samples were boiled for 5 min. Twenty microliters of each reaction was analyzed by SDS-PAGE on 10% gel and stained with Coomassie Blue R-250.

Hydrolysis of p-nitroanilide chromogenic substrates

The steady-state kinetic analysis of hydrolysis of several commercially available chromogenic substrates by the wild type and the mutant enzymes was performed at room temperature in TBS buffer containing 1 mg/mL BSA and 0.1% PEG 8000. The concentration of chromogenic substrates ranged from 1 to 2,000 μM, and the concentration of enzymes ranged from 0.5 nM to 50 nM, depending on the k_{cat} values.

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 equation mentioned above using ENZFITTER software (R.J. Leatherbarrow, Elsevier, Biosoft). All of the data presented are the average of at least three to six independent measurements ±SD.

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