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## [総説] Rational Design to Develop Antithrombotic Drugs from the Relationship between the Structure and Function of $\alpha$ thrombin

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## Rational Design to Develop Antithrombotic Drugs from the Relationship between the Structure and Function of $\alpha$ -thrombin

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### ABSTRACT

Many protease inhibitors in the circulating blood inhibit the activity of  $\alpha$ -thrombin. Inhibition of  $\alpha$ -thrombin by antithrombins is attributable to the characteristics of structural relationships between  $\alpha$ -thrombin and antithrombins. The characteristic structures of  $\alpha$ -thrombin include loop 60, exosites 1 and 2, hydrophobic pocket, primary specificity pocket, and active site (catalytic triad). Functions of these structures have been clarified by enzyme-substrate reaction and X-ray and NMR-crystallographic analyses. For developing antithrombin drugs that is safely and effectively used for clinical treatment of thrombotic diseases, the precise understandings of relationship between structure and function of  $\alpha$ -thrombin are required. Although it has been evidenced that clot-bound thrombin plays more important role in pathophysiological conditions than native (free) thrombin, the structure and function of clot-bound thrombin remains to be clarified. Understanding the structure and function of clot-bound thrombin would provide a potential therapeutic strategy to develop novel antithrombin drugs, which control the activity of the clot-bound thrombin in the circulating blood. In this review paper, first of all we summarized the structure-function relationships based on the studies on native thrombin and mutant thrombin. Secondary, we summarized the mechanism for thrombin receptor activation by  $\alpha$ -thrombin and thrombin receptor agonist peptide (TRAP). Finally, we demonstrated our results of the studies on the characteristics of clot-bound thrombin. *Ryukyu Med. J.*, 25(3,4) 95~109, 2006

Key words: antithrombotic drug, native thrombin, clot-bound thrombin, structure-function relationship.

### INTRODUCTION

$\alpha$ -thrombin is not only a key substance in coagulation system of haemostasis but also a trigger to repair or regenerate impaired tissue. On the other hand,  $\alpha$ -thrombin plays a harmful role in the formation of thrombus in the patients with thrombotic disease. The characteristic structure

of  $\alpha$ -thrombin is related to its multifunction. Since the structure of  $\alpha$ -thrombin was elucidated, the relationship between the structure and function of  $\alpha$ -thrombin has been investigated to make clear the mechanism for the development of multifunction. In addition, the structure-oriented antithrombotic drug has been developed on the basis of definite information obtained from the investigation.

Table 1 Comparison of the Relative Thrombin Inhibitory Effectiveness of  $\alpha_1$ -Antitrypsin, Antithrombin III, and  $\alpha_2$ -Macroglobulin

Inhibitor	Plasma concn ( $\mu\text{M}$ ) <sup>a</sup>	2nd-order rate constant ( $\text{M}^{-1}\text{min}^{-1}$ )	Rel effectiveness as a thrombin inhibitor
$\alpha_2$ -Macroglobulin	3.11	$2.93 \times 10^4$	1
$\alpha_1$ -Antitrypsin	42.4	$6.51 \times 10^3$	3.03
Antithrombin III	4.46	$3.36 \times 10^5$	15.64

<sup>a</sup> Calculated from data given by Kueppers & Black (reference 1).  
Rel indicated "relative" in this table (modified from reference 2).

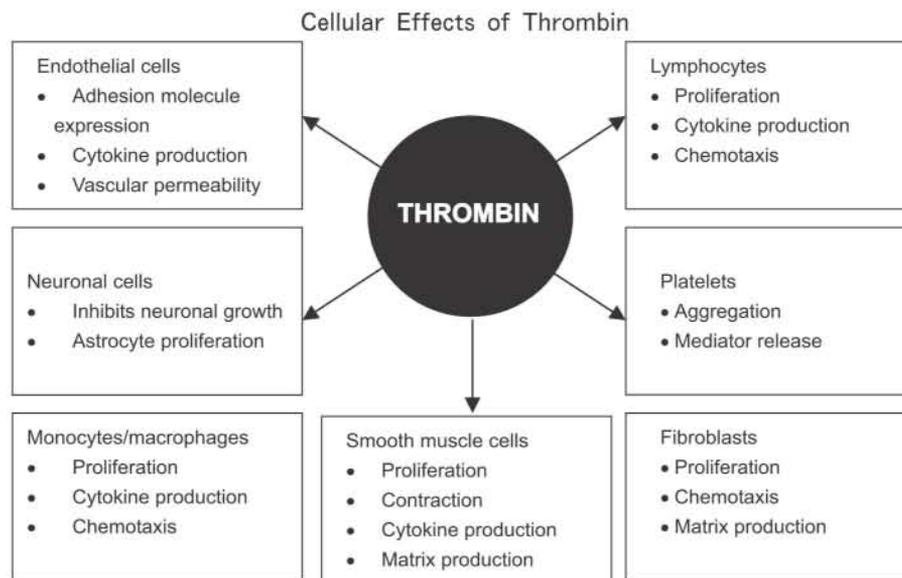


Fig. 1 Cellular effects of thrombin.

Thrombin stimulates a variety of cellular effects, most of which are critical to tissue repair. (This figure was modified from reference 3)

Crystallography contributed as a powerful tool to elucidate the structures of a variety of molecules. In addition, the information from crystallography of the complex of  $\alpha$ -thrombin with different antithrombotic drugs in comparison with that of  $\alpha$ -thrombin alone clarified the detailed and precise structure of  $\alpha$ -thrombin.

In this paper, we reviewed the characteristic structures of  $\alpha$ -thrombin, which can react with thrombomodulin (TM), thrombin activatable fibrinolysis inhibitor (TAFI), protein C, the various cells and antithrombotic drugs. Furthermore, the modes of actions of different antithrombotic drugs were reviewed from the aspect of structural interaction of  $\alpha$ -thrombin with antithrombotic drugs.

## MULTIFUNCTION OF $\alpha$ -THROMBIN

Many protease inhibitors in the circulating blood

inhibit the activity of  $\alpha$ -thrombin.  $\alpha_1$ -antitrypsin,  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M) and antithrombin III (AT-III) are typical representatives of protease inhibitors in the circulating blood. It has been shown that antithrombin activity of AT-III is approximately 5 times stronger than that of  $\alpha_1$ -antitrypsin (Table 1)<sup>1,2</sup>. The strong inhibition of  $\alpha$ -thrombin by AT-III is attributable to the characteristic structural relationship between  $\alpha$ -thrombin and AT-III. On the other hand,  $\alpha$ -thrombin is a key substance to maintain the physiological homeostasis in relation to haemostasis and tissue repair. Additionally,  $\alpha$ -thrombin can induce proliferation and migration of the vascular smooth muscle cells (VSMC) derived from various animals (Fig. 1)<sup>3</sup>.  $\alpha$ -thrombin promotes inflammation by mobilizing a number of different cell types including monocytes, macrophages and neutrophils.  $\alpha$ -thrombin also participates in the processes of tissue repair by

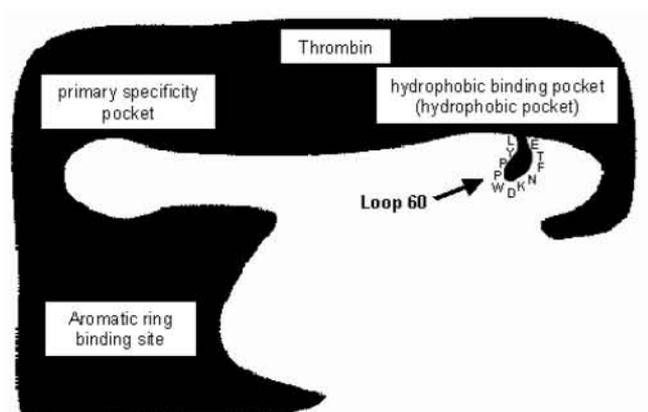


Fig. 2 The active site and loop 60 of thrombin.  
(This figure was modified from reference 37)

stimulating expression of endothelial cell surface adhesion molecules and enhances vascular permeability and leucocyte extravasation. Furthermore,  $\alpha$ -thrombin stimulates proliferation of fibroblasts and epithelial cells. These cellular effects of  $\alpha$ -thrombin were typically observed in the blood vessel wall with atherosclerotic lesions and repaired tissue (Fig. 1)<sup>3)</sup>.

$\alpha$ -thrombin can react with fibrinogen (Fgn), TM, TAFI, protein C and the different cells (vascular endothelial cells, VSMC, platelets, etc.). The appearance of a multifunction of  $\alpha$ -thrombin is dependent upon its intricate structure. Until the present time, to control the enhanced activity of  $\alpha$ -thrombin under the various pathological conditions, some antithrombin drugs with the different actions have been released.

### THE ROLE OF LOOP 60 IN THE STRUCTURE OF $\alpha$ -THROMBIN

Asp<sup>189</sup> exists at the bottom of primary specific pocket near by the active site of  $\alpha$ -thrombin. The loop between Leu<sup>60</sup> and Glu<sup>61</sup> is composed of nine amino acids (Tyr<sup>60A</sup>-Pro<sup>60B</sup>-Pro<sup>60C</sup>-Trp<sup>60D</sup>-Asp<sup>60E</sup>-Lys<sup>60F</sup>-Asn<sup>60G</sup>-Phe<sup>60H</sup>-Thr<sup>60I</sup>, Fig. 2)<sup>36)</sup> was termed loop 60, which is lacking in another serine protease, chymotrypsin. The loop 60 guards the  $\alpha$ -thrombin against the inactivation induced by bovine pancreatic trypsin inhibitor (BPTI) by preventing BPTI from entering the primary specific pocket. Consequently, BPTI cannot inhibit the activity of  $\alpha$ -thrombin.

In addition, Bonniec *et al* investigated whether each amino acid of loop 60 influenced the function of  $\alpha$ -thrombin<sup>4)</sup>. That is, they investigated whether

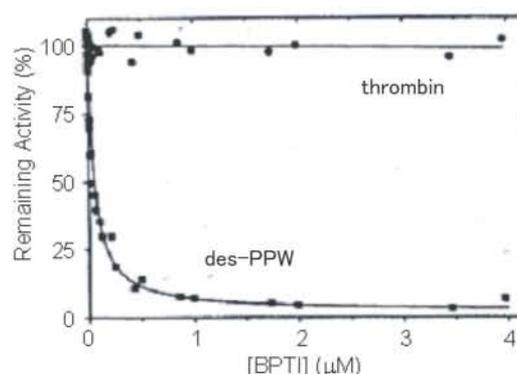


Fig. 3 Significance of amino acids composition in loop 60 on the function of thrombin. The thrombin with des-PPW loop 60 was inhibited by BPTI. Steady state velocities of H-D-Val-Leu-Arg-pNA hydrolysis by des-PPW (■) or thrombin (●) in the presence of increasing concentrations of BPTI. The rates of cleavage are expressed as percentage of the remaining activities. Des-PPW is almost totally inhibited by 50 nM BPTI, whereas thrombin is still unaffected by 4  $\mu$ M BPTI. (This figures was cited from reference 4)

the amino acids of Pro<sup>60B</sup>, Pro<sup>60C</sup> and Trp<sup>60D</sup> in the loop 60 could interact with Fgn, TM, protein C, AT-III or kunitz type inhibitor. AT-III inhibition of the des-PPW thrombin (mutant thrombin with the deletion of Pro<sup>60B</sup>, Pro<sup>60C</sup> and Trp<sup>60D</sup>) decreased by 100-fold, as compared with that of wild type thrombin. The activation of protein C by des-PPW thrombin decreased by 30-fold, as compared with that of wild type thrombin. After the lag time of the reaction of the des-PPW thrombin with Fgn, the levels of the released fibrinopeptide B (FPB) reached to an equal level of the released fibrinopeptide A (FPA). The des-PPW thrombin, however, had a strong affinity for BPTI (Fig. 3)<sup>4)</sup>. These results show that the amino acids of Pro<sup>60B</sup>, Pro<sup>60C</sup> and Trp<sup>60D</sup> in the loop 60 play an important role in the development of substrate specificity of  $\alpha$ -thrombin.

### ACTIVE SITE OF $\alpha$ -THROMBIN

It has been known for a long time that hirudin, a protein found in the leech (*Hirudo medicinalis*), had antithrombin activity. Until the present time, different antithrombin agents derived from the leech have been developed. In many cases, the production of peptide inhibitor that binds to the recognition site on  $\alpha$ -thrombin for fibrinogen was very important to develop antithrombin agents.

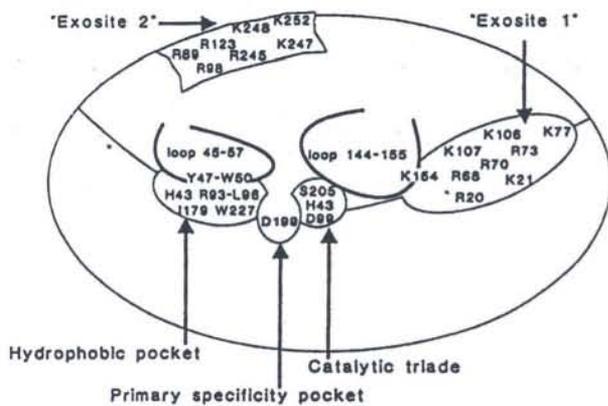


Fig. 4 Schematic representation of human  $\alpha$ -thrombin. Two insertion loops (Leu 45-Asn 57 and Leu 144-Gly 155) project over a narrow and deep canyon in which resides the active site, consisting of the catalytic triade Ser-His-Asp flanked by the specificity pocket and an extended hydrophobic pocket. A number of positively charged residues are concentrated at two poles of the molecule, on both sides of the active site. The positive patch located close to the carboxy terminal B chain helix, referred to as "anion binding exosite 2", is involved in heparin binding. The positive patch occurring on the surface of the loop 65-76 surrounded by other positively charged residues, referred to as "anion binding exosite 1", interacts with the hirudin carboxy-terminal tail. Numbering of amino acids is based on  $\alpha$ -thrombin. (This figure was cited from reference 7)

Therefore, various peptide inhibitors were synthesized. Crystallographic analysis has shown that some synthetic peptide inhibitors formed a complex with  $\alpha$ -thrombin by binding to a separate position from the active site. These synthetic peptide inhibitors, however, could inhibit the activity of  $\alpha$ -thrombin. From the above-mentioned results, the binding of synthetic peptide inhibitor to different sites from the active site indirectly induce a conformational change of the active site, thereby inhibiting  $\alpha$ -thrombin activity. Accordingly, the synthetic peptide inhibitors can bind to the specific peptide-recognition site, which are connected to the active site of  $\alpha$ -thrombin. For example, there are two basic amino acid-rich regions, which are positively charged. Therefore, they are called anion binding exosite 1 and 2, respectively. The exertion of functional activity of the active site (exosite) needs its conformational change following the conformational change of the different site<sup>5)</sup>. Structural analysis of  $\alpha$ -thrombin using autoantibodies in

patients with recurrent thrombosis showed that the variable region I of  $\alpha$ -thrombin was important for interaction of  $\alpha$ -thrombin with protein C or  $\alpha_1$ -antitrypsin<sup>6)</sup>. This indicates the importance of conformational change of  $\alpha$ -thrombin for binding to protein C or  $\alpha_1$ -antithrombin.

## EXOSITES 1 AND 2 OF $\alpha$ -THROMBIN

To analyze the structure-function relationship of  $\alpha$ -thrombin, the complexes of antibodies against mutant and native (wild) thrombins with their respective thrombins or the complexes of different protease inhibitors with thrombins were produced and used *in vitro* experiments.  $\alpha$ -thrombin can react with diverse substrates, and these reactions were dependent upon which site of  $\alpha$ -thrombin (active site, active site cleft or exosite) the substrates reacted with. Insertion of a loop into the surroundings on Trp<sup>50</sup> and Trp<sup>148</sup> at the top of active site cleft changed the selectivity of the substrate. This suggests that active site cleft of  $\alpha$ -thrombin is a possible determinant to select a substrate. In addition, macromolecules including Fgn, heparin co-factor II, TM and glycoprotein Ib (GPIb) reacted with a large exosite (anion binding exosite 1) of  $\alpha$ -thrombin that was composed of basic amino acids. These macromolecules reacted with the confined and specific residue in the exosite 1. In the structure of the exosite 1, some subsites exist and overlap each other.

Anion binding exosite 2 exists near the  $\beta$  chain helix in the C-terminal of  $\alpha$ -thrombin and reacts with the active fragment 2 of prothrombin and the chondroitin sulfate in the molecule of heparin and TM. Therefore, there are two distinctive anion binding exosites in the structure of  $\alpha$ -thrombin and the conformation change of  $\alpha$ -thrombin occurs after some substances relating to the coagulation-fibrinolysis system and platelet function bind to either anion binding exosite 1 or 2. Such change of conformation provides the  $\alpha$ -thrombin with the development of substrate specificity (Fig. 4)<sup>7)</sup>. The functional interaction between anion binding exosite 1 and 2, however, has not been obviously shown. Although it had been unclear how conformational change of active site occurred after the binding of ligand to these exosites, the two exosites were reportedly to independently affect the active site and the binding of ligand to one exosite could affect the binding of ligand to another exosite<sup>8)</sup>. On

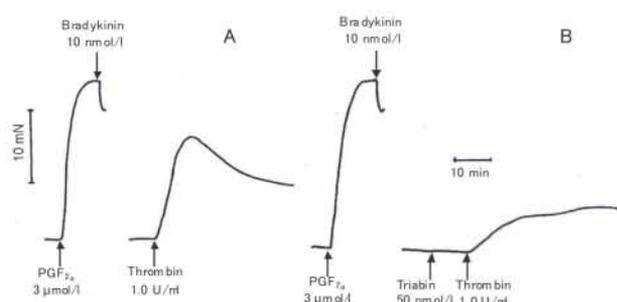


Fig. 5 Contractile responses of endothelium-denuded ring segments of porcine pulmonary arteries to PG  $F_{2\alpha}$  and thrombin (10 nM).

After preincubation of the vessel with triabin the contractile response to thrombin was delayed and reduced. In the absence of endothelium relaxant responses to bradykinin were markedly reduced. (This figure was cited from reference 11)

the other hand, it has been shown how the direct thrombin inhibitor interacts with the fibrinogen-binding site (exosite 1) and the active site pocket of  $\alpha$ -thrombin. The C-terminal group of hirudin binds to the exosite 1, whereas its N-terminal group binds to the active site pocket. In addition, the C-terminal groups of hirugen and hirulog bind to the exosite 1. The N-terminal group of hirulog binds to the active site pocket.

Argatroban was a direct synthetic thrombin inhibitor and its N-terminal group could bind to the active site pocket of  $\alpha$ -thrombin<sup>9)</sup>. Argatroban that directly binds to the active site pocket is a useful antithrombotic drug for the therapy of some thrombotic diseases. However, argatroban has a side effect with bleeding. The efficacy of argatroban is generally accepted, but the side effect of it must be excluded. Direct antithrombin drugs including argatroban, then, have been investigated in relation to the clinical availability and usefulness<sup>10)</sup>. To establish an effective administration of the direct thrombin inhibitor for treatment of pathophysiological conditions such as rethrombosis, the precise mechanism of inhibitory action of thrombin inhibitor must be understood.

Triabin, a thrombin inhibitor derived from the insect, inhibits  $\alpha$ -thrombin by binding to the anion binding exosite 1. This inhibitor also inhibits  $\alpha$ -thrombin-induced contraction of porcine pulmonary arteries without endothelial cells (Fig. 5)<sup>11)</sup>. Furthermore, triabin inhibits the  $\alpha$ -thrombin-induced mitogenic activity of bovine coronary

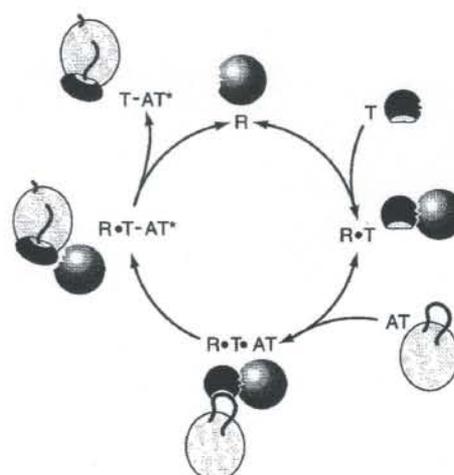


Fig. 6 Model for exosite I-dependent interactions of a regulatory protein accompanying thrombin inactivation by antithrombin.

Binding of a regulatory protein (R) to thrombin (T) through exosite I is proposed to allow reversible binding of antithrombin (AT) to the thrombin active site to form a regulatory protein-thrombin-antithrombin complex (R·T·AT). Inactivation of exosite is proposed to accompany either the thrombin-antithrombin chemical reaction or the subsequent serpin conformational change, which is shown as a single irreversible reaction. Dissociation of the regulatory protein is postulated to occur subsequent to the rate-limiting irreversible formation of the stable product complex (T·AT\*). (This figure was cited from reference 13)

VSMC. This suggests that the anion binding exosite 1 plays an important role in the cell response induced by  $\alpha$ -thrombin<sup>11)</sup>. The binding of heparin to antithrombin was accelerated by the heparin-dependent modification of side-chain of arginine in the reactive center of antithrombin<sup>12)</sup>. It has been reported that inactivation of  $\alpha$ -thrombin by antithrombin was dependent upon the inactivation of its regulatory exosite 1 (Fig. 6)<sup>13)</sup>.

### $\alpha$ -THROMBIN AND SODIUM ION

Since  $\alpha$ -thrombin was reported to be a sodium-dependent allosteric enzyme, a new strategy to inhibit and control the activity of  $\alpha$ -thrombin has been explored. Sodium ion binds to  $\alpha$ -thrombin as an effector that induces conformational change of  $\alpha$ -thrombin structure, resulting in the change of specificity of the catalytic activity. In addition, some components of the complement system and vitamin K-dependent proteases have the same

Table 2 Summary of Thrombin Inhibition Constants by Antithrombin<sup>a</sup>

thrombin	$\kappa_2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$		rate enhancement	optimal heparin concn (units/mL)
	without heparin	with heparin		
wild type	0.85 ± 0.23	648 ± 16.5	759	0.5
R93, 97, 101A	0.87 ± 0.27	2.1 ± 0.3	2.4	10.0
R93A	0.87 ± 0.23	126 ± 6.3	144	1.0
R97A	0.99 ± 0.27	208 ± 13.0	209	0.5–1.0
R101A	0.72 ± 0.09	56 ± 10.4	78	2.5
R93, 97A	0.95 ± 0.27	18 ± 2.2	19	5.0
R93, 101A	0.63 ± 0.18	7.4 ± 0.8	12	5.0
R97, 101A	0.68 ± 0.24	12 ± 0.6	18	5.0

<sup>a</sup>Second-order association rate constants ( $\kappa_2$ ) for antithrombin inhibition of wild-type and R93, 97, 101A thrombins in the absence and presence of heparin are shown. The optimal concentration of heparin is indicated in the last column. All values are the average of at least three independent measurements with  $\pm$  SE values.

(This table was cited from reference 17)

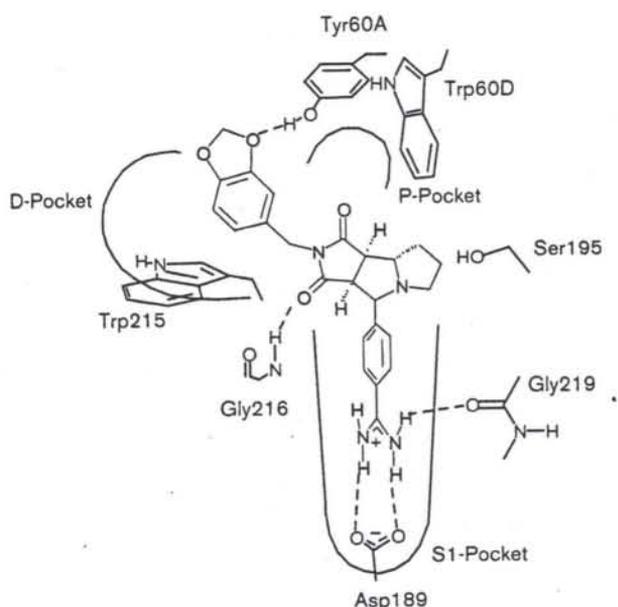


Fig. 7 Schematic representation of the complex formed by thrombin and ent-1, according to the X-ray crystal structure.

(This figure was cited from reference 16)

mechanism as  $\alpha$ -thrombin exerts its protease activity due to sodium-dependent control<sup>15</sup>. It has been determined that the sodium-dependent allosteric regulation observed in the  $\alpha$ -thrombin was dependent upon the Tyr<sup>235</sup> in its structure<sup>15</sup>.

### THE SIGNIFICANCE OF Arg<sup>93</sup>, Arg<sup>97</sup>, Arg<sup>101</sup> IN STRUCTURE OF $\alpha$ -THROMBIN

A thrombin inhibitor with a strong inhibition against thrombin activity and highly specific selectivity for  $\alpha$ -thrombin would be the most useful for clinical treatment. The stable dicyclic compound, a

thrombin inhibitor, was produced by the structure-based design. This compound has a powerful inhibitory activity against  $\alpha$ -thrombin, but does not have so high specificity for  $\alpha$ -thrombin. Later, a nonpeptide and high selective thrombin inhibitor was designed and synthesized by taking into the consideration of strength on binding between two molecules ( $\alpha$ -thrombin and ent-1) (Fig. 7)<sup>15,16</sup>.

To refine the mechanism on the development of the specificity and inhibitory activity of  $\alpha$ -thrombin, the mutant thrombin was synthesized. That is, the Args (Arg<sup>93</sup>, Arg<sup>97</sup>, Arg<sup>101</sup>) in the structure of  $\alpha$ -thrombin were substituted by Ala in the mutant thrombins (R-A thrombins). It has been shown that the inhibition against the wild thrombin by the combination of antithrombin with heparin was enhanced with the increase of heparin. However, the inhibition against the mutant R-A thrombins was not enhanced with the increase of heparin. Accordingly, it was shown that Args in the structure of  $\alpha$ -thrombin were essential for the development on the accelerated activity of antithrombin by heparin. In addition, the affinity of chondroitin sulfate of TM or protein C to the mutant R-A thrombins was decreased in comparison to that to the wild thrombin. Collectively, the enhanced activity of TM or protein C by  $\alpha$ -thrombin was produced by the affinity of heparin or chondroitin sulfate to  $\alpha$ -thrombin, but such enhanced activity was not expressed in the cases of the mutant R-A thrombins. From the data on the inhibitory constants of antithrombin in the presence of heparin, the mutant R-A thrombins were ranked in the order of the strong affinity for thrombin inhibitor. Consequently, Arg<sup>101</sup> (as R98 by

Table 3 Effect of peptides derived from the human thrombin receptor on the aggregation of platelets from various animal species

Species	Aggregation <sup>a</sup>	Shape change
Human	87 ± 5	YES
Baboon	70 ± 4	YES
African green monkey	57 ± 9	YES
Rhesus monkey	68 ± 12	YES
Rabbit	3 ± 1	YES
Dog	0	YES
Pig	14 ± 2	YES
Rat	0	NO
Hamster	0	YES
Guinea pig	65 ± 13	YES

<sup>a</sup> Platelet rich plasma was challenged by up to 800  $\mu$ M SFLLRN-NH<sub>2</sub>, SFLLRN-NH<sub>2</sub> or SFLLRNPNDKYEPF peptides and aggregation and shape change monitored. Aggregation values are expressed as the maximal extent of aggregation observed  $\pm$  the standard deviation. Results are from 3-6 separate experiments for each species.

(This table was cited from reference 23)

human  $\alpha$ -thrombin B chain numbering) was the most important residue for the inhibition by antithrombin with heparin (Table 2)<sup>17</sup>. On the other hand, Arg<sup>97</sup> (as R93 by human  $\alpha$ -thrombin B chain numbering) was the most important residue on the TM-dependent interaction of  $\alpha$ -thrombin with antithrombin and on the chondroitin sulfate-dependent affinity of TM for  $\alpha$ -thrombin<sup>17</sup>. These results demonstrated that the Arg residues in anion binding exosite 2 were related to the diversity of  $\alpha$ -thrombin-induced reactions and a multifunction of  $\alpha$ -thrombin.

### EXOSITE 1 OF $\alpha$ -THROMBIN FOR ACTIVATION OF PLATELET

From the analysis on interaction of the exosite 1 with platelet, it was clarified that the interaction of  $\alpha$ -thrombin with GPIIb in platelet was caused by the hydrolysis of thrombin receptor (protease activated receptor 1, PAR1). Once PAR1 is activated by its cleavage by  $\alpha$ -thrombin,  $\alpha$ -thrombin no longer binds to PAR1. Therefore, the decrease in interaction of  $\alpha$ -thrombin with PAR1 promotes the binding of  $\alpha$ -thrombin to GPIIb. In addition, the 238-290 segment in the domain of N-terminus of GPIIb $\alpha$  and the exosite 1 with cation of  $\alpha$ -thrombin were participated in the interaction of  $\alpha$ -thrombin with platelet. From the studies on the significance of GPIIb $\alpha$  269-287 segment, it was shown that the interaction of

$\alpha$ -thrombin with platelet resulted from the electrostatic force of attraction between GPIIb $\alpha$  269-287 segment of GPIIb $\alpha$  238-290 and exosite 1. Furthermore, it was suggested that Pro<sup>280</sup> in GPIIb had an important role in the interaction of  $\alpha$ -thrombin with GPIIb<sup>18</sup>.

### $\alpha$ -THROMBIN AND ANTITHROMBIN

The active site of  $\alpha$ -thrombin is shaped as a cleft where a substrate binds. The residues of the substrate are labeled as Pi, ..., P3, P2, P1, P1', P2', P3', ..., Pj from the scissile P1-P1' bond toward the N- and C-termini, respectively. Their respective binding subsites of  $\alpha$ -thrombin are labeled as Si, ..., S3, S2, S1, S1', S2', S3', ..., Sj. The cleavage is catalyzed between S1 and S1'. The activation of antithrombin induced by heparin depends on the pentasaccharide in heparin. The complex of the pentasaccharide with antithrombin was crystallized and the structure of the complex was analysed<sup>19</sup>. Consequently, S<sub>2</sub> and S<sub>3</sub> in the  $\alpha$ -thrombin subsites were essential to recognize P<sub>2</sub> and P<sub>3</sub> residues in antithrombin under the condition of native conformation. Under the condition of active conformation in antithrombin, the other residues than P<sub>2</sub> and P<sub>3</sub> interacted with some proteases.  $\alpha$ -thrombin could react with the antithrombin under both conditions of the heparin-induced active conformation and the native conformation. S2 and S3 subsite residues in

Table 4 Effect of alanine-substituted TRAPs on  $[Ca^{2+}]_i$  in neutrophils

Residue substituted	Aggregation <sup>a</sup>
Ser1'	0*
Phe2'	0*
Leu3'	0*
Leu4'	177 ± 15
Arg5'	0*
Asn6'	97 ± 12

Neutrophils were obtained from donor, loaded with Fura-2 and challenged with a series of alanine-substituted peptides (200  $\mu$ M). The activity of the peptides is expressed as a percentage of the peak  $[Ca^{2+}]_i$  elevation observed with unsubstituted TRAP (mean  $\pm$  s.e.m.; n=3). Amino acid residues in TRAP are labelled with primed numbers that indicate their position in the cleaved thrombin receptor. Therefore, Ser1' is equivalent to Ser42 of the deduced amino acid sequence.

\*No  $Ca^{2+}$  response was observed with these alanine-substituted peptides.

(This table was cited from reference 26)

$\alpha$ -thrombin are essential to form an intermediary complex of  $\alpha$ -thrombin with antithrombin, which determines to enter the pathway of accelerated or decelerated reaction for  $\alpha$ -thrombin substrate<sup>20</sup>.

### ACTIVATION OF $\alpha$ -THROMBIN RECEPTOR AND CELL RESPONSE

$\alpha$ -thrombin is a biologically multifunctional enzyme and plays an important role in cell response in the process of tissue repair after haemostasis. Thrombin receptor expressed in different cells is a family of the proteolytically-activated receptor (PAR). The cloning of thrombin receptor cDNA from human megakaryocyte mRNA showed that the functional constitution belonged to G-protein linked receptor family<sup>21</sup>. Thrombin receptor agonist peptide (TRAP) of tethered ligand to PAR is a useful tool to investigate whether the informative signals for cell proliferation in tissue repair is mediated via thrombin receptor<sup>22</sup>. The platelet aggregation using the PAR-activated peptide derived from thrombin receptor was investigated in a variety of animals. The platelets from rabbit, dog, pig and hamster were activated by the PAR-activated peptide in the same manner as by TRAP, but the mode of activation was different (Table 3)<sup>23</sup>. The heterogeneity of platelet was observed among the various animals. Accordingly, it was considered that the reactivity for the peptide was different from the platelets and cells derived from various animals. A potential thrombin receptor antagonist derived

from tethered ligand agonist peptides, 3-mercaptopropionyl-Phe-Cha (cyclohexylalanyl)-Cha-Arg-Lys-Pro-Asn-Asp-Lys-amide (Mpa-peptide), inhibited the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and Na<sup>+</sup>/H<sup>+</sup> exchanger induced by  $\alpha$ -thrombin or the peptide-containing Ser-Phe-Leu-Leu-Arg (SFLLR)<sup>24</sup>. In the process of platelet activation, it was shown that a small amount of  $\alpha$ -thrombin produced a peptide as the tethered ligand, and then this peptide reacted with the thrombin receptor and the platelet was activated. If the thrombin receptor was split at the different sites of the thrombin receptor by the various proteases, the platelet was not activated. That is, it was essential for the activation of thrombin receptor that Arg<sup>41</sup>-Ser<sup>42</sup> peptide bond in thrombin receptor was hydrolysed. Serine proteases such as trypsin and plasmin could split the Arg<sup>41</sup>-Ser<sup>42</sup> peptide bond in the same manner as  $\alpha$ -thrombin. Chymotrypsin, however, splits a site far from Arg<sup>41</sup> in the amino acid sequence of thrombin receptor. Accordingly, chymotrypsin-digested thrombin receptor could not react with the  $\alpha$ -thrombin, but could react with the thrombin receptor agonist peptide (TRAP)<sup>25</sup>. The thrombin receptor of neutrophil which lacks proteolytic activation is related to chemotactic function of neutrophil. In addition, TRAP itself could not induce neutrophil chemotaxis. Wild-type thrombin and Ser<sup>195</sup>-Ala mutant thrombin did not increase the calcium concentration in neutrophil from the different animals, but the TRAPs with the substitution of Ala (Leu4' and Asn6', amino acids of TRAP are numbered 1'

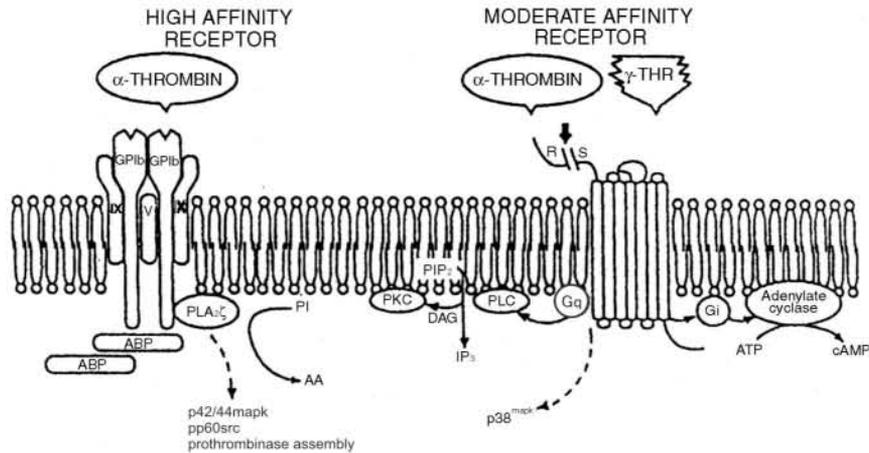


Fig. 8 The two receptor model for  $\alpha$ -thrombin-induced platelet activation.

The high affinity receptor is shown as the supercomplexed form comprising GPIIb $\alpha$ , GPIIb $\beta$ , GPIX and GPV in the ratio of 2:2:2:1 and the receptor probably exists as a dimer of this supercomplex. Recent data show that this receptor is coupled to PLA $_2$  $\zeta$  isoform (14-3-3 protein) and signal transduction initiated by this receptor involves p42/44<sup>mapk</sup> and pp60<sup>src</sup> and results in prothrombinase expression. The STDR is the moderate affinity receptor and is coupled to adenylyl cyclase and PLC leading to inositol phosphate production and activation of p38<sup>mapk</sup> but association of the latter to the cytoskeleton apparently involves GPIb. Extensive further studies are necessary to elucidate the signal transduction pathways coupled to each of the two receptors.

(This figure was modified from reference 27)

through 6' from N-terminal) did increase the calcium concentration (Table 4)<sup>26</sup>. It was rarely reported, on the contrary, that the thrombin receptor of neutrophil was proteolytically activated and that TRAP could react with the receptor and increased the calcium concentration in the neutrophils. From these controversial results, two types of thrombin receptor seemed to exist in the neutrophil<sup>26</sup>. Two different types of thrombin receptors (the high affinity receptor and the moderate affinity receptor) actually existed in platelets<sup>27</sup>. The high affinity receptors belonged to GPIIb $\alpha$ , GPIIb $\beta$ , GPIX and GPV, which were coupled to phospholipase A2 (PLA2), whereas the moderate affinity receptor was activated by the cleavage of the receptor by proteases, had the transmembrane structure, and was coupled to PLC (Fig. 8)<sup>27</sup>.

Tissue factor-dependent thrombin generation, platelet activation and thrombus formation were developed at the locus of the mechanical impairment of blood vessel and the rupture of atheromatous plaque of atherosclerotic disease. In these pathological conditions, an antithrombotic therapy must be taken into the consideration for prevention from the thrombin generation and the activation of thrombin

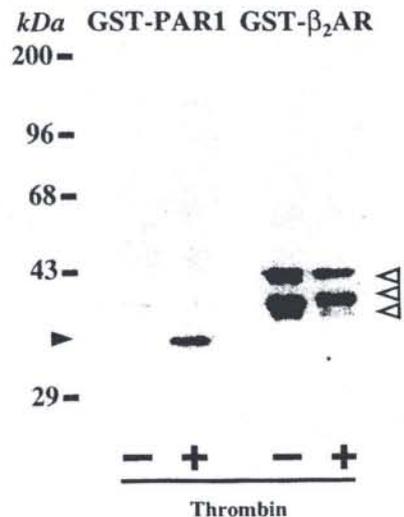


Fig. 9 Substrate specificity of the 33-kDa protein kinase. Extracts prepared from unstimulated or thrombin (10 nM for 1 min)-stimulated platelets were incubated with GST-PAR-1 immobilized to glutathione Sepharose 4B. Proteins bound to GST-PAR-1 were examined by the renaturable protein kinase assay using GST-PAR-1 or GST- $\beta_2$ AR. Closed arrowhead indicates the 33-kDa protein kinase and open arrowheads indicate protein kinases that preferably phosphorylate GST $\beta_2$ AR. (This figure was modified from reference 29)

Table 5 Second-Order Association Rate Constants ( $k_2$ ) for PAI-1 Inactivation of Different Thrombin Derivatives in the Absence and Presence of Heparin or Vitronectin<sup>d</sup>

Residue substituted	-co-factor <sup>a</sup>	+heparin <sup>b</sup>	+vitronectin <sup>c</sup>
$\alpha$ -thrombin	$(7.9 \pm 0.5) \times 10^2$	$(1.6 \pm 0.2) \times 10^5$	$(1.9 \pm 0.3) \times 10^5$
R93, 97, 101A	$(1.5 \pm 0.1) \times 10^3$	$(1.3 \pm 0.2) \times 10^3$	$(1.2 \pm 0.1) \times 10^5$
$\gamma$ -thrombin	$(7.9 \pm 0.7) \times 10^1$	$(2.2 \pm 0.3) \times 10^4$	$(5.9 \pm 2.1) \times 10^3$
meizothrombin	$(4.6 \pm 0.3) \times 10^2$	$(3.6 \pm 0.1) \times 10^2$	$(1.4 \pm 0.2) \times 10^4$
thrombin + TM4-6	$(6.7 \pm 0.5) \times 10^1$	$(1.9 \pm 0.4) \times 10^4$	$(8.3 \pm 1.6) \times 10^3$
thrombin + sTM	$(1.1 \pm 0.3) \times 10^2$	ND <sup>e</sup>	$(1.0 \pm 0.2) \times 10^3$

<sup>a</sup>The  $k_2$  values ( $M^{-1}s^{-1}$ ) were determined by a discontinuous assay method using either the chromogenic substrate Spectrozyme PCa or the fluorogenic substrate Tosyl-GPA-amc. <sup>b</sup>The  $k_2$  values were determined in the presence of optimal concentration of heparin (50-200 nM) by a discontinuous assay method. <sup>c</sup>The  $k_2$  values were determined in the presence of 500-1000 nM vitronectin by a discontinuous assay method. <sup>d</sup>Around 40-80% of enzyme activity was inhibited for calculation of the inactivation rate constants. Prolonged incubation and/or incubation at high concentrations of PAI-1 resulted in complete inhibition of all thrombin variants. All values are an average of at least three independent measurements  $\pm$  SD. <sup>e</sup>ND, not determined. (This table was cited from reference 30)

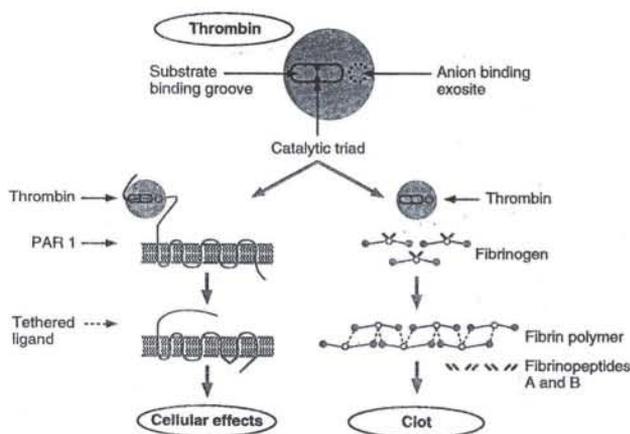


Fig. 10 Structure and function of thrombin.

This diagram shows a schematic representation of thrombin and its interaction with the cellular PAR-1 (protease activated receptor-1) receptor and fibrinogen. The interaction between thrombin and PAR-1 initiates a variety of cellular effects that are summarized in Fig. 1. The interaction between thrombin and fibrinogen is the final step of the coagulation cascade leading to the fibrin clot. Thrombin has several discrete sites. The anion binding exosite of thrombin lies within the deep groove and binds its substrates. The substrate binding sites within this deep groove orientates thrombin's substrates (PAR-1 or fibrinogen) enabling cleavage at the catalytic site at an Arg/Ser susceptible bond. (This figure was cited from reference 3)

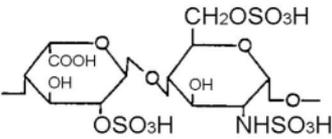
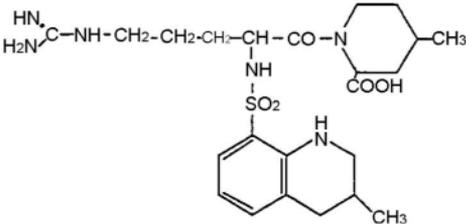
receptor by  $\alpha$ -thrombin. For a successful therapeutic strategy, it is essential to adequately control the activity of clot-bound thrombin, thrombin-induced

activation of protein C, the enhanced function of thrombin receptor and tissue factor-induced generation of  $\alpha$ -thrombin<sup>28</sup>). Identification of a protein kinase that phosphorylates PAR-1 had not been succeeded until 1999. In the year of 2000, a 33-kDa Ser/Thr kinase was finally identified as the protein kinase, which phosphorylated the amino acid in the cytoplasmic tail of PAR-1 (Fig. 9)<sup>29</sup>. PAR-1, characterized as the G-protein linked receptor existed in the endothelial cell of blood vessel, neutrophil, monocytes/macrophage, smooth muscle cells, lymphocytes, platelet and fibroblast (Fig. 10)<sup>31</sup>. Recently, it was reported that PAR-3 existed in platelet of mouse.

### $\alpha$ -THROMBIN AND PLASMINOGEN ACTIVATOR INHIBITOR-1

Plasminogen activator inhibitor (PAI) inhibits the activity of plasminogen activator (PA). Until the present time, PAIs were classified into four types based on the physicochemical properties of them. Of these, PAI-1 was studied into great details about enzymatic characteristics and the pathophysiological significance. From these studies, PAI-1 affected the reactivity with the substrate of  $\alpha$ -thrombin in the presence of the co-factor. That is, the exosites 1 and 2 contributed to the reactivity to substrate of  $\alpha$ -thrombin, which was modulated by PAI-1 with the co-factor. Heparin binding to the exosite 2 enhanced the reactivity of PAI-1 by inhibiting the

Table 6 Antithrombin Agents

	Heparin	Argatroban	Warfarin
Structure	 <p>main component</p>		
Molecular weight (daltons)	5,000~30,000	509	308
Mode of action	Inhibition of thrombin and Xa resulting from the formation of the complex of heparin with AT-III	Selective specific inhibition due to the direct binding to the active site of thrombin	Antagonist of vitamin K, the production of PIVKA (without the coagulant activity)
Administration	Injection	Injection	Per Os

(This table was modified from reference 37)

binding of PAI-1 to  $\alpha$ -thrombin. On the other hand, vitronectin accelerated the inactivation of PAI-1 by  $\alpha$ -thrombin, and both exosites 1 and 2 did not relate to this accelerated inhibition. The binding site of PAI-1 may exist in the exosite 1 or near the exosite 1 of  $\alpha$ -thrombin. It has been thought that the occupation of the exosite 1 by TM prevented, in part, from abruptly inactivating  $\alpha$ -thrombin by PAI-1 in the presence of vitronectin (Table 5)<sup>30</sup>.

#### THE INTERACTION OF THE DOMAIN IN $\alpha$ -THROMBIN WITH TM, TAFI, PROTEIN C AND Fgn

At first, the domains in  $\alpha$ -thrombin which interacted with TM, TAFI and protein C have been determined. The mapping of functional residues in the structure of  $\alpha$ -thrombin demonstrated that 11 residues in the exosite 1 mediated to interact TM with  $\alpha$ -thrombin and that some residues at the shallow space in the active site cleft mediated to react with TAFI, and that some residues at the deep space in the active site cleft mediated to react with protein C. The residues mediating to react with TM overlapped extensively with those to react with fibrinogen. However, the residues to react with protein C lied at the distant space from those to react with TAFI<sup>31</sup>.

#### SYNTHETIC ANTITHROMBIN AGENTS

$\alpha$ -thrombin is involved in coagulation, fibrinolysis, wound healing, migration and proliferation of inflammatory cells, development and maintenance of brain. The studies on the relationship between the structure and function of  $\alpha$ -thrombin were promoted and progressed by many researchers in biochemistry, physiology and pharmacology, resulting from the development of the novel and synthetic antithrombin agents<sup>32</sup>. Hirudin and hirugen, at first, derived from natural source, were released as antithrombin agents. Next, the synthetic hirudin peptide without immunologic action was then developed as a synthetic antithrombin agent derived from hirudin or hirugen<sup>33</sup>.

The site-directed mutagenesis was applied to define the role of basic amino acid residues in the anion binding exosite 1 in the formation of the thrombin-AT-III complex, thrombin-protease nexin 1 (PN1) complex and thrombin-heparin co-factor II inhibitor complex. The exosite 1 did not have an important role in the interaction of  $\alpha$ -thrombin with AT-III irrespective of the coexistence of heparin. However, the exosite 1 of  $\alpha$ -thrombin had an important role in the interaction of heparin co-factor II with  $\alpha$ -thrombin in the presence of heparin or dermatan sulfate. The connection of hirudin-like

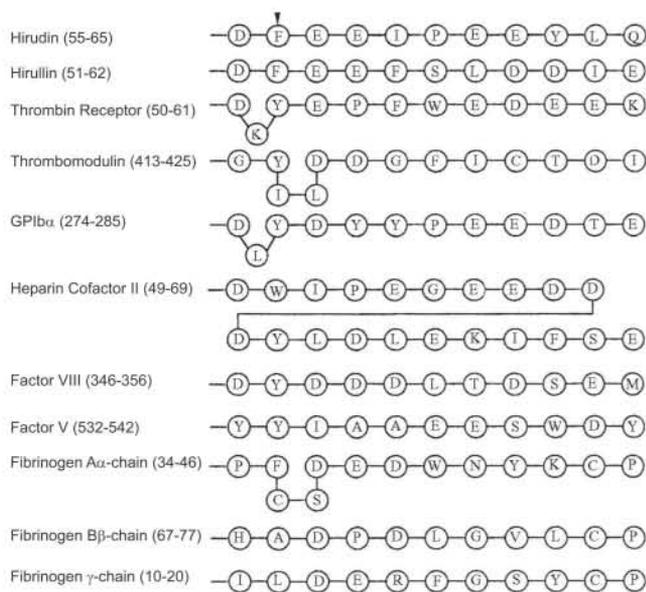


Fig. 11 Alignment of acidic domains from various macromolecules involved in binding to thrombin's anion-binding exosite I.

Alignment of hirudin, hirullin, thrombin receptor, and thrombomodulin is based on the comparison of crystallographic structures of each ligand complexed to  $\alpha$ -thrombin. Common to all of the structures was a Phe or Tyr (*arrowhead*) that binds in a pocket formed by Phe<sup>34</sup>, Arg<sup>73</sup>, and Thr<sup>74</sup>. Also, salt-bridge interactions with Arg<sup>73</sup>, Arg<sup>76</sup>, and Arg<sup>77</sup> were considered for these alignments. The remaining alignments were arbitrary and, where possible, based on importance of Phe and Tyr that bind in the pocket formed by Phe<sup>34</sup>, Arg<sup>73</sup>, and Thr<sup>74</sup>. (This figure was cited from reference 34)

domain in heparin cofactor II with the exosite 1 was essential for the interaction between heparin cofactor II with  $\alpha$ -thrombin (Fig. 11)<sup>34</sup>. It has been shown that various monoclonal antibodies against  $\alpha$ -thrombin did not induce conformational change of the exosite 1. In addition, such a lack of effect of the monoclonal antibodies against  $\alpha$ -thrombin was also observed in the structure of  $\alpha$ -thrombin irrespective of the coexistence of sodium, which was an allosteric regulator of  $\alpha$ -thrombin. From these results, it was suggested that the direct allosteric linkage between the catalytic site and the exosite 2 of  $\alpha$ -thrombin existed<sup>35</sup>. It has been known that the hirudin segment had an affinity for the exosite 1 and that the linker existed in the active site cleft. This linker, however, did not directly interact with the active site of  $\alpha$ -thrombin. On the other hand, the insertion of some peptides that bound to the exosite 1 into the structure of antithrombin agent could enhance the

antithrombin activity<sup>36</sup>. Therefore, it is important to design an antithrombin agent that binds simultaneously to both the active site and exosite 1. The anticoagulants used in clinical field are now divided into three classes based on their different mechanisms of action as follows: 1) indirect antithrombin agents, 2) direct antithrombin agents, 3) antagonist of vitamin K (Table 6). Heparin belongs to the first group. Antithrombin agents, which belong to the second group, are further divided into two subclasses as follows: a) active site-oriented antithrombin agents, b) thrombin inhibitor with two kinds of the inhibitory functions. Argatroban belongs to the subclass a) and hirudin and antithrombin agents derived from hirudin belong to the subclass b). The development of argatroban has accompanied with the elucidation of the structure of active site itself and surrounding structure of the active site in  $\alpha$ -thrombin<sup>37</sup>.

## MONOCLONAL ANTIBODIES AGAINST $\alpha$ -THROMBIN

Monoclonal antibodies against any different parts of  $\alpha$ -thrombin are useful to analyze the structure of  $\alpha$ -thrombin. In addition to the usefulness of monoclonal antibodies for analyzing the structure of  $\alpha$ -thrombin, the monoclonal antibody was useful to elucidate the function of different parts in  $\alpha$ -thrombin. We produced the monoclonal antibody against native thrombin, mAbGE<sub>12</sub>, which bound to C-terminal region and surrounding region of  $\alpha$ -thrombin<sup>38</sup>. In addition, we also produced the monoclonal antibody against diisopropylfluorophosphate (DFP)-thrombin, mAbcc2, which competed with the activity of argatroban. Thus, mAbcc2 bound to the surrounding structure of the primary specific pocket in  $\alpha$ -thrombin<sup>39</sup>. These monoclonal antibodies are useful for the inhibition of  $\alpha$ -thrombin-induced biological activity for the analysis of structure and for elucidation of the functions of  $\alpha$ -thrombin.

## SUMMARY

The development of the active site-oriented antithrombin agent accelerated the progression of studies on the analyses of the structure of  $\alpha$ -thrombin. Because the multiple biological and physiological functions of  $\alpha$ -thrombin were depend-

ent upon the structural characteristics of it, the information obtained from analysis of the structure enabled the design and development of the novel synthetic antithrombin agents. At the present time, many proposals for the development of the effective, novel antithrombin agent from clinicians and researchers in the clinical field are offered to researchers in the pathophysiological and pharmacological field. These proposals, thus, induce the initiation of the attractive and original research of pharmacology and basic medicine. The proper elucidation of the interaction between the  $\alpha$ -thrombin and antithrombin agent has progressively been obtained from the investigation of the relationship between the structure of  $\alpha$ -thrombin and the effectiveness of antithrombin agent in the clinical field. The structure-function relation of  $\alpha$ -thrombin has been exclusively studied using the free (native) thrombin until the present time. Under the physiological conditions, the free (native) thrombin barely exists in the circulating blood and the fibrinogen, the substrate of the free (native) thrombin, exists abundantly, which results in the fibrin formation. Consequently, the structure and function of the clot-bound thrombin must be adequately studied in order to develop the novel antithrombin agent. It is expected that all of the thrombin in the circulating blood of patients with the thrombotic diseases and the atherosclerotic stenosis of blood vessel is the clot-bound thrombin but not the free (native) thrombin. The structure and function of the clot-bound thrombin has been gradually revealed by the investigation in our laboratory<sup>40-43</sup>. It has been elucidated that the clot-bound thrombin has an important role in the tissue repair, transformation of VSMC and regeneration after the hemostasis<sup>44-48</sup>. The studies on the pathophysiological significance of the clot-bound thrombin have just been started in the clinical field. In the near future, the interrelationship between the structure of clot-bound thrombin and the effectiveness of antithrombin agent will be clarified.

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