CMLS, Cell. mol. life sci. 53 (1997) 731–736
1420-682X/97/090731-06 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Mini-Review

Strategies for development of novel antithrombotics: modulating thrombin's procoagulant and anticoagulant properties

S. W. Hall[a,*], C. S. Gibbs[b] and L. L. K. Leung[a]

[a]Division of Hematology, Department of Medicine, Stanford University Medical School, 300 Pasteur Drive, Stanford (California 94305-5112, USA), Fax +1 650 7231 269, e-mail: HF.LKL@Forsythe.Stanford.edu
[b]Gilead Sciences, Foster City 650 (California, USA)

Abstract. Thrombin is a serine proteinase that can interact with a large number of diverse macromolecular substrates, which results in either a procoagulant or anticoagulant effect. These divergent properties are physiologically regulated by the endogenous protein thrombomodulin. This review summarizes recent work on a variety of methods used to exploit the allosteric nature of the enzyme. The procoagulant and anticoagulant functions of thrombin can be modulated by sodium binding, site-directed mutagenesis, and a small synthetic molecule. Modulation of thrombin's intrinsic properties represents a novel approach to the development of unique antithrombotic agents.

Key words. Thrombin; structure; mutagenesis; protein C; allosteric.

Introduction

At the site of vascular injury, a cascade of events is initiated which ultimately leads to the conversion of prothrombin to the active serine proteinase thrombin. Thrombin in turn is then capable of catalysing a wide variety of reactions that have both procoagulant and anticoagulant effects. Procoagulant activities include the conversion of fibrinogen to fibrin, activation of the clotting cascade factors V, VIII, XI, XIII, activation of the platelet thrombin receptor leading to platelet aggregation, and activation of a plasma carboxypeptidase (TAFI) leading to inhibition of fibrinolysis [1–3]. Thrombin's anticoagulant activity stems from its interaction with thrombomodulin on the endothelial cell surface which causes a dramatic shift in substrate specificity. Thrombomodulin-bound thrombin preferentially activates protein C (APC), which then inactivates Factors Va and VIIIa and attenuates further generation of thrombin [4]. The importance of this endogenous anticoagulant pathway is illustrated in patients with protein C deficiency or with a mutation in their Factor V (Factor V Leiden) which renders it resistant to inactivation by APC; patients with these defects are at higher risk for developing thromboembolic disease. In addition to this negative feedback loop, thrombin activity is also limited by its primary inhibitor, antithrombin III (ATIII), as well as heparin cofactor II, α^2-macroglobulin, and protease nexin I.

The ability of thrombin to interact with such a wide diversity of macromolecules has generated a tremendous amount of research into the structure-function relationships of thrombin. These studies have been greatly aided by the X-ray crystallographic structure of thrombin-inhibitor complexes, which have revealed distinct domains in the molecule which provide for its

* Corresponding author.

diversity and specificity of interactions [5–7]. The central importance of thrombin in thrombosis, hemostasis, and cell activation is perhaps underscored by the recent discovery in mice that targeted disruption of the prothrombin gene is embryonically lethal [ref. 8, Sun, Degan, personal communication]. In addition, a recent Medline search using the keyword "thrombin" reveals more than 5000 references in the past 5 years alone. This review aims to highlight recent work on the modulation of thrombin's procoagulant and anticoagulant functions.

Thrombin structure

After cleavage by the prothrombinase complex, prothrombin is converted to the active molecule α-thrombin which consists of an A and B chain linked by a disulphide bond. Crystallographic studies of α-thrombin reveal the molecule to be a compact, globular prolate ellipsoid. The active site consists of the classic serine proteinase catalytic triad, H43, D99 and S205 (human thrombin B chain numbering), which is located in a deep narrow canyon-like cleft bounded by the primary specificity pocket (D199) and two insertion loops (L45-N57, L144-G155) (fig. 1A). Unique to thrombin are the presence of two positively charged, anion-binding domains removed from the active site which are critically important in mediating many of thrombin's macromolecular interactions. These sites have been termed exosite I and exosite II (fig. 1B and 1C). Exosite I has been implicated in binding to fibrin(o-gen), hirudin, thrombomodulin, the thrombin receptor, thrombin aptamer and heparin cofactor II [9–13]. Exosite II mediates binding of heparin and subsequent complex formation with antithrombin III (ATIII) and heparin cofactor II. Prothrombin fragment F2 interacts with this site [14–16], and recently both exosites have been implicated in the activation of Factors V and VIII [17].

Functional mapping of the charged and polar surface residues on thrombin involved in fibrinogen recognition, protein C activation and thrombomodulin binding reveals that they are all clustered on the hemisphere of the molecule that contains the active site cleft and exosite I [18]. No functional residues were located on the opposite hemisphere. Interestingly, the A chain of α-thrombin is localized to this backside, and most likely the linker to the F2 proenzyme domain as well [19]. Based on these data, it appears that thrombin is a polarized enzyme, with one face devoted to its proteolytic activity and multiple substrate interactions, while the opposite face, which contains the linker domain which is cleaved during activation of the enzyme, may contain important residues involved in the recognition and activation of the proenzyme by the prothrombinase complex.

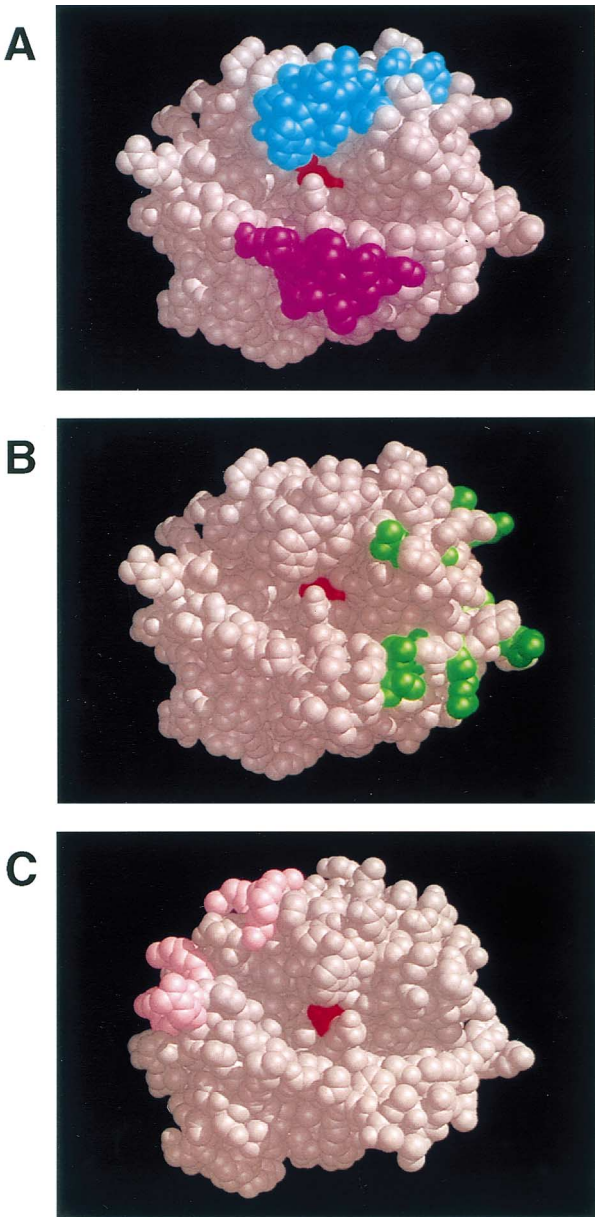

Figure 1. Localization of structural domains on the surface of thrombin. Models generated based on the coordinates of the PPACK-thrombin complex with PPACK removed using MidasPlus (University of California, San Francisco). (*A*) Space filling model of human thrombin in the standard orientation, looking directly into the active site cleft. Catalytic residue S205 is coloured red in all models. Insertion loop L45-N57 located above the active site is coloured light blue. Insertion loop L144-G155 is below and coloured magenta. (*B*) Exosite I residues lying to the right of the active site are in green. Residues depicted are R20, K21, R68, R70, R73, K77, K106, K107, and K154. (*C*) Exosite II residues lying above and to the left of the catalytic domain are depicted in pink. Residues comprising this domain include R89, R93, R98, R123, R170, R174, R180, R245, K247, K248, K252, some of which lie on the backside of the molecule

Thrombomodulin regulation of thrombin

The primary endogenous modulator of thrombin activity is the endothelial cell surface protein thrombomodulin [4, 20]. Binding of thrombin to this molecule results in a dramatic switch in substrate specificity: fibrinogen clotting activity is reduced to $<5\%$, whereas the catalytic efficiency for protein C activation is increased 10,000-fold. The molecular basis for this switch is multifactorial. Thrombomodulin acts as a competitive inhibitor of fibrinogen clotting by binding through exosite I, inhibiting access of fibrinogen. In addition, this interaction blocks platelet activation. In the free molecule, there are postulated repulsive interactions between E25 and E202 of thrombin and two aspartate residues in protein C (located at the P3 and P3′ positions), thus making it a poor substrate for thrombin. Binding of thrombomodulin to thrombin causes a conformational change in thrombin at the S3 and S3′ sites, relieving this repulsive interaction and allowing efficient cleavage of protein C. Recently, however, this mechanism has been challenged by Vindigni and coworkers [21]. Using a library of small chromogenic substrates to probe the S1, S2, and S3 specificity sites of thrombin, only a modest effect of thrombomodulin binding was observed (maximum 15-fold enhancement of substrate specificity). They propose that thrombomodulin exerts its effects on thrombin specificity by influencing the conformation of protein C in the thrombin-thrombomodulin-protein C ternary complex, converting protein C into a better substrate for thrombin. The thrombin-thrombomodulin interaction therefore serves as potent trigger for physiological anticoagulant responses.

Allosteric regulation of thrombin by sodium

The ability to modulate thrombin-substrate interactions allosterically ions was described by Di Cera and colleagues when they demonstrated that sodium could enhance the specificity of thrombin toward a small chromogenic substrate [22, 23]. Thrombin was found to equilibrate between two forms, termed fast and slow which referred to their ability to cleave the substrate. Subsequent studies have shown this allosteric switch to be regulated by a sodium binding site in the molecule,

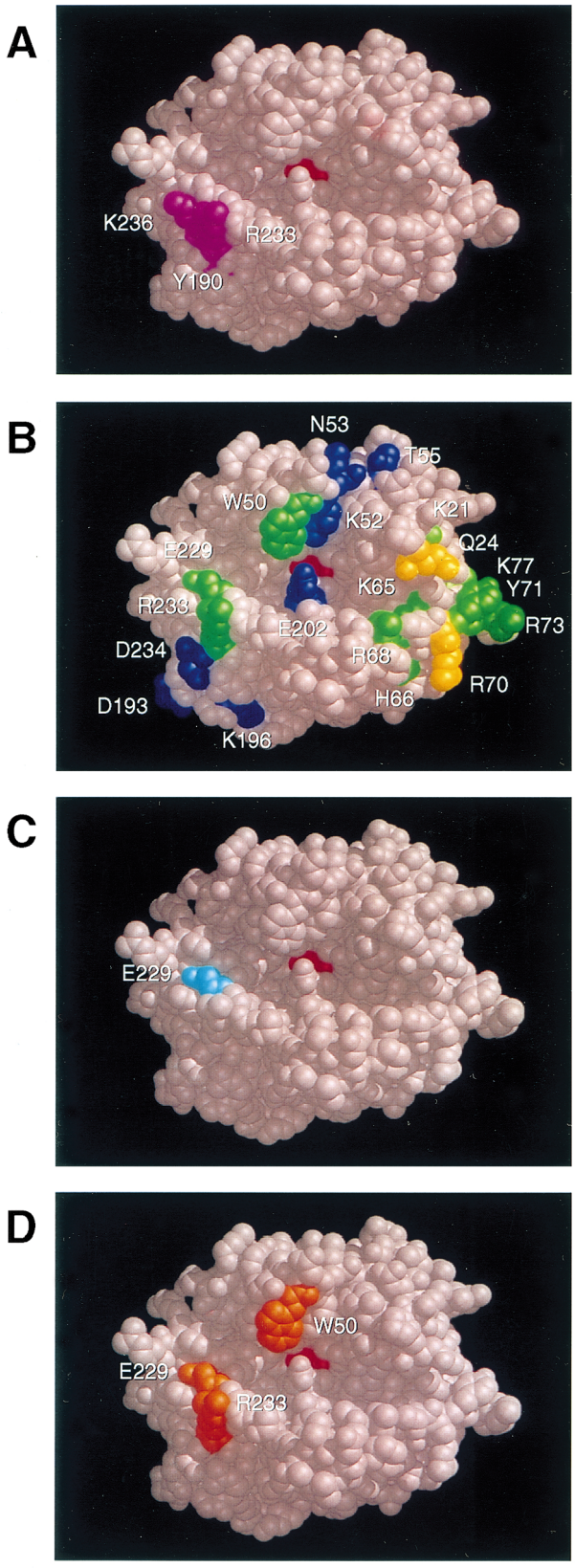

Figure 2. Localization of functional domains on the surface of thrombin. Catalytic residue S205 is coloured red in all models. (*A*) The sodium binding site of thrombin. Sodium binding residues Y190, R233 and K236 are coloured magenta. (*B*) Residues involved in the recognition of fibrinogen and thrombomodulin-dependent protein C activation. Blue residues are involved only in fibrinogen recognition, yellow residues are involved only in TM-dependent protein C activation, and green residues are involved in both functions. (*C*) Residue E229, the site mutated to generate the protein C activators (PCAs) E229A and E229K-thrombin. (*D*) Residues mediating the direct interaction with ATIII are coloured gold (W50, E229, R233).

which is conserved in other serine proteinases in the coagulation and complement pathways. The sodium-bound, fast form is procoagulant in that it has a higher specificity for fibrinogen than the sodium-free, slow form, which more specifically activates protein C [24]. The fast form also binds hirudin and its N-terminal fragments with higher affinity [25].

The sodium binding loop has been identified by X-ray crystallography and spans residues 227–239 of the molecule [26]. Sodium is bound via the carbonyl oxygens of Y190, R233 and K236 (fig. 2A). Mutation of K236 results in disruption of sodium binding, converting thrombin to the anticoagulant, slow form [27]. In addition, the ability of the carbonyl oxygen atom of K236 to contribute to this sodium binding site is highly dependent on the identity of the neighbouring residue 237. Comparison of 54 different serine proteinases shows that residue 237 is either proline or tyrosine in 46 of the enzymes. Those enzymes with Y237, like thrombin and Factor Xa, can be allosterically modulated by sodium, whereas those with P237, like plasmin and trypsin, do not show regulation by sodium. The role of this residue in sodium binding is probably dominant, in that the thrombin mutant Y237P is devoid of allosteric regulation [28]. This intriguing observation suggests that proteinases with P237 could be engineered by site-directed mutagenesis to endow them with sodium-induced allosteric enhancement of catalytic activity.

The ability of sodium binding at one site to influence substrate interactions at sites located 5–20 Å away suggests that there are other residues linked to the allosteric transitions of the enzyme. A map of residues energetically linked to the slow → fast transition is beginning to emerge [29]. These include the sodium binding loop, the W50 insertion loop, E202 and E25 located near exosite I. This core of residues provides the basis for the transmission of structural changes from one site of the molecule to the other areas involved in thrombin's multiple substrate interactions.

Modulation of thrombin by protein engineering

Wu and coworkers first discovered that the procoagulant and anticoagulant properties of thrombin could be dissociated by site-directed mutagenesis [30]. We recently carried out a systematic study of the surface residues of thrombin in order to map the binding sites required for fibrinogen clotting and thrombomodulin-dependent protein C activation [18]. The hypothesis was that if these epitopes represented distinct sites on the molecule, it might be possible to develop specific inhibitors which could block clotting activity while preserving anticoagulant activity. This class of inhibitor would offer advantages over current inhibitors which nonspecifically inhibit both the procoagulant and anticoagulant properties of thrombin.

To map the surface of thrombin functionally, 77 charged and polar surface residues determined to be accessible to a solvent probe of 1.4 Å were replaced by alanine (alanine scanning mutagenesis). This collection of mutants was then screened for their ability to clot fibrinogen (procoagulant) and activate protein C (anticoagulant) in the presence and absence of thrombomodulin. All residues that were functionally identified mapped to the hemisphere of the molecule containing the active site and exosite I (fig. 2B). In addition, there is considerable topographical overlap between these functional residues, with many being involved in both procoagulant and anticoagulant activities (K21, W50, K65, H66, R68, Y71, R73, K77, K106/K107, E229, R233). However, a small number of mutations were found to have predominant isolated effects on either procoagulant activity (K52, N53/T55, D193/K196, E202, D234) or anticoagulant activity (Q24, R70).

When the functional mutations were analysed in terms of their relative substrate specificity for fibrinogen clotting and protein C activation, it was discovered that four mutants (W50, K52, E229, R233) displayed a significant substrate shift in favour of protein C activation. These mutants, representing a novel class of anticoagulant, have been termed protein C activators (PCAs). The prototype PCA, E229A, which displayed a 22-fold switch in substrate specificity (compared to wild-type), was shown to function as an endogenous protein C activator in Cynomolgus monkeys, causing reversible anticoagulation as determined by prolongation of the partial thromboplastin clotting time without consumption of fibrinogen, activation of platelets or prolongation of the bleeding time [31].

In order to identify the optimal PCA, the four mutants which were identified in the initial screen were subjected to saturation mutagenesis in which each residue was substituted with all 19 natural amino acids [32]. This screen revealed that E229K had a catalytic efficiency for FPA release of 0.4% compared to wildtype, while maintaining 50% efficiency for protein C activation, representing a 130-fold switch in substrate specificity (fig. 2C). In addition, E229K demonstrated diminished platelet aggregation (18-fold) and increased resistance to ATIII (33-fold and 22-fold in the presence and absence of heparin). Like E229A, E229K produced reversible anticoagulation in Cynomolgus monkeys without platelet consumption or aggregation, or fibrinogen, Factor V or VIII consumption. Because of the superior specificity and ATIII resistance of E229K thrombin relative to E229A thrombin, an equivalent effect was achieved with 20% of the infusional dose (0.5 $\mu g\ kg^{-1}\ min^{-1}$ vs. 2.5 $\mu g\ kg^{-1}\ min^{-1}$) with a longer half-life (60 min vs. 40 min). Again, template bleeding times at the peak of anticoagulation were not prolonged.

The antithrombotic properties of E229K have been tested in a baboon AV shunt thrombosis model [33]. In this model, the effect of anticoagulants can be tested under both high shear stress conditions (primarily platelet deposition) and low shear conditions (fibrin deposition). At an infusion rate of 0.1 μg kg^{-1} min^{-1}, E229K reduced platelet deposition ~50% and fibrin deposition ~80% demonstrating in vivo effectiveness in both arterial and venous thrombosis. In contrast to the direct thrombin inhibitor hirudin and GPIIb-IIIa antagonists in the same model, there was no effect on simplate bleeding times during the infusion, suggesting that PCAs may have a superior therapeutic index in terms of bleeding risk.

Because E229 is located near the sodium binding loop, it is possible that mutations at this site could disrupt sodium binding, thereby locking thrombin in the slow conformation and shifting its substrate specificity toward protein C activation. The carboxyl oxygens of glutamate 229 do form a salt bridge with the side chain nitrogen atom of lysine 236, whose carbonyl oxygen is involved in sodium binding. While plausible, several observations suggest that substitution at E229 has effects beyond disruption of sodium binding. Firstly, the change in catalytic efficiency (k_{cat}/K_m) toward fibrinopeptide A (FPA) cleavage is reduced ~260-fold in E229K, compared with ~8-fold reduction for the slow form of thrombin. Secondly, there is no change in affinity (K_m 4.4 nM vs. 3.5 nM for WT) of E229K for thrombomodulin, whereas the slow form of thrombin has a 4.4-fold decrease in affinity (14 nM vs. 3.2 nM) [24]. Lastly, E229 is observed in the crystal structure of thrombin-FPA to be in van der Waals contact with the P5 glycine residue, which is highly conserved in FPA from higher vertebrates, suggesting this interaction is important in thrombin's specificity for fibrinogen.

Small molecule modulation of thrombin

An ideal anticoagulant would be an agent that specifically inhibits thrombin's procoagulant activity, has no increased risk of bleeding, a broad therapeutic profile, good oral bioavailability, and relatively long half-life. Alternatively, given the allosteric nature of the thrombin molecule, small molecules that could modulate its intrinsic procoagulant and anticoagulant properties could be exploited to enhance the endogenous protein C pathway (much like the PCAs described above). Berg and coworkers [34] recently screened ~8300 synthetic compounds for agents that would enhance thrombin-dependent activation of protein C. Those agents with a positive effect were then examined for effects on fibrinogen cleavage. Compounds with both activities were examined for structural similarities, and additional analogues synthesized. Using this approach, they identified a compound, LY254603, with a 10-fold increase in APC generation, with a >95% decrease in clotting activity, representing ~200-fold shift in substrate specificity.

In order to understand the mechanism behind this effect, they examined the effects of calcium, sodium, and protein C mutants in their assay. While calcium can induce conformational changes in protein C affecting its activation by thrombin, no dependency on calcium concentration was found. Sodium concentration also had no effect, indicating that the compound does not alter the transition between the slow and fast forms of thrombin. However, activation of a protein C mutant, D167F, was not enhanced, while wild-type and another mutant, D172K, were activated ~10-fold faster. Mutation of these aspartate residues of protein C (located in the P3 and P3′ positions) are felt to relieve the repulsive interactions between thrombin and protein C, similar to the effect of thrombomodulin. Therefore, LY254603 simulates in part the action of thrombomodulin. However, LY254603 was still able to enhance APC generation ~3-fold in the presence of saturating amounts of thrombomodulin, suggesting additional allosteric effects on thrombin. Although its in vivo antithrombotic properties remain to be determined, LY254603 represents the prototype small molecule thrombin modulator.

Thrombin regulation by ATIII

The primary inhibitor of thrombin in vivo is ATIII, although this reaction is relatively slow in the absence of heparin. In the presence of heparin, a trimolecular complex is formed, resulting in a ~1000-fold increase in inhibition rate. The heparin binding site on thrombin was mapped previously to exosite II [14, 15], and a recent study demonstrated that W50 is involved in the thrombin-ATIII reaction [35]. Because ATIII directly modulates thrombin activity and is involved in the clearance of active thrombin in vivo, the collection of alanine mutants was screened to identify mutations involved in this interaction. Only three residues were identified that are likely to mediate the direct interactions of thrombin with ATIII: W50, E229, and R233 [36]. These residues cluster around the active site cleft (fig. 2D), and most likely contact the substrate loop of ATIII. This observation is surprising in the light of thrombin's well known ability to interact with macromolecular substrates through its exosites. Unlike other macromolecular substrates, ATIII in the absence of heparin is recognized much like a small tripeptidyl substrate. The resistance of thrombin mutants substituted at residue E229 to inhibition by ATIII probably explains why E229K has a prolonged half-life in plasma [32].

Summary

Thrombin is an allosteric enzyme with both procoagulant and anticoagulant activities. These activities can be

modulated by sodium, thrombomodulin, site-directed mutagenesis, and by a small synthetic molecule. In conjunction with the crystal structure of the molecule, site-directed mutagenesis has identified key residues mediating these interactions. E229K-thrombin is a potent protein C activator which demonstrates anticoagulant and antithrombotic efficacy in vivo. Similarly, the small molecule modulator LY254603 can shift thrombin's substrate specificity in vitro. These agents, which exploit the endogenous protein C anticoagulant pathway, may offer therapeutic advantages over today's currently used antithrombotics.

1 Fenton J. W. (1995) Thrombin functions and antithrombotic intervention. Thromb. Haemost. **74:** 493–498
2 Mann K. G. (1994) Prothrombin and thrombin. In: Hemostasis and Thrombosis, 3rd edition, pp. 184–199, Coleman R. W., Hirsh J., Marder V. J. and Salzman E. W. (eds), Lippincott, Philadelphia
3 Bajzar L., Mavel R. and Nesheim M. E. (1995) Purification and characterization of TAFI, a thrombin-activatable fibrinolysis inhibitor. J. Biol. Chem. **270:** 1447–1452
4 Esmon C. T. (1995) Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J. **9:** 946–955
5 Bode W., Mayr I., Baumann U., Huber R., Stone S. R. and Hofsteenge J. (1989) The refined 1.9 A crystal structure of human α-thrombin: interaction with D-Phe-Pro-Arg-chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. EMBO J. **8:** 3467–3475
6 Grütter M. G., Priestle J. P., Rahuel J., Grossenbacher H., Bode W., Hofsteenge J. et al. (1990) Crystal structure of the thrombin-hirudin complex: a novel mode of serine protease inhibitor. EMBO J. **9:** 2361–2365
7 Rydel T. J., Ravichandran K. G., Tulinsky A., Bode W., Huber R., Roitsch C. et al. (1990) The structure of a complex of recombinant hirudin and human *alpha*-thrombin. Science **249:** 277–280
8 Xue J., Wu Q., Westfield L. A., Tuley E. A., Lu D., Zhang Q. et al. (1996) A genetically engineered mouse model of total prothrombin deficiency. Blood **88,** Suppl 1: 468a
9 Fenton II J. W., Olson T. A., Zabinski M. P. and Wilner G. D. (1988) Anion-binding exosite of human α-thrombin and fibrin(ogen) recognition. Biochemistry **27:** 7106–7112
10 Stone S. R. and Hofsteenge J. (1986) Kinetics of the inhibition of thrombin by hirudin. Biochemistry **25:** 4622–4628
11 Sheehan J. P., Wu Q., Tollefsen D. M. and Sadler J. E. (1993) Mutagenesis of thrombin selectively modulates inhibition by serpins heparin cofactor II and antithrombin III. Interaction with the anion-binding exosite determines heparin cofactor II specificity J. Biol. Chem. **268:** 3639–3645
12 Liu L. W., Vu T. K. H, Esmon C. T. and Coughlin S. R. (1991) The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. J. Biol. Chem. **266:** 16977–16980
13 Li W. X., Kaplan A. V., Grant G. W., Toole J. J. and Leung L. L. K. (1994) A novel nucleotide-based thrombin inhibitor inhibits clot-bound thrombin and reduces arterial platelet thrombus formation. Blood **83:** 677–682
14 Sheehan J. P. and Sadler J. E. (1994) Molecular mapping of the heparin-binding exosite of thrombin. Proc. Natl Acad. Sci. USA **91:** 5518–5522
15 Gan Z. R., Li Y., Chen Z., Lewis S. D. and Shafer J. A. (1994) Identification of basic amino acid residues in thrombin essential for heparin-catalyzed inactivation by antithrombin III. J. Biol. Chem. **269:** 1301–1305
16 Arni R. K., Padmanabhan K., Padmanabhan K. P., Wu T. P. and Tulinsky A. (1993) Structure of the noncovalent complexes of human and bovine prothrombin fragment 2 with human PPACK-thrombin. Biochemistry **32:** 4727–4737
17 Esmon C. T. and Lollar P. (1996) Involvement of thrombin anion-binding exosites 1 and 2 in the activation of factor V and factor VIII. J. Biol. Chem. **271:** 13882–13887
18 Tsiang M., Jain A. K., Dunn K. E., Rojas M. E., Leung L. L. K. and Gibbs C. S. (1995) Functional mapping of the surface residues of thrombin. J. Biol. Chem. **270:** 16854–16863
19 van de Locht A., Stubbs M. T., Bauer M. and Bode W. (1996) Crystallographic evidence that the F2 kringle catalytic domain linker of prothrombin does not cover the fibrinogen recognition exosite. J. Biol. Chem. **271:** 3413–3416
20 LeBonniec B. F. and Esmon C. T. (1991) Glu-192 [to] Gln substitution in thrombin mimics the catalytic switch induced by thrombomodulin. Proc. Natl Acad. Sci. USA **88:** 7371–7375
21 Vindigni A., White C. E., Komines E. A. and Di Cera E. (1997) Energetics of thrombin-thrombomodulin interaction. Biochemistry **36:** 6674–6681
22 Wells C. M. and Di Cera E. (1992) Thrombin is a Na^+-activated enzyme. Biochemistry **31:** 11721–11730
23 De Cristofaro R. and Di Cera E. (1992) Modulation of thrombin-fibrinogen interaction by specific ion effects. Biochemistry **31:** 257–265
24 Dang Q. D., Vindigni A. and Di Cera E. (1995) An allosteric switch controls the procoagulant and anticoagulant activities of thrombin. Proc. Natl Acad. Sci. USA **92:** 5977–5981
25 Ayala Y. M., Vindigni A., Nayal M., Spolar R. S., Record M. T. and Di Cera E. (1995) Thermodynamic investigation of hirudin binding to the slow and fast forms of thrombin: evidence for folding transitions in the inhibitor and protease coupled to binding. J. Molec. Biol. **253:** 787–798
26 Di Cera E., Guinto E. R., Vindigni A., Dang Q. D., Ayala Y. M., Wuyi M. et al. (1995) The Na^+ binding site of thrombin. J. Biol. Chem. **270:** 22089–22092
27 Dang Q. D., Guinto E. R. and Di Cera E. (1997) Rational engineering of activity and specificity in a serine protease. Nature Biotechnology **15:** 146–149
28 Dang Q. D. and Di Cera E. (1996) Residue 225 determines the Na^+-induced allosteric regulation of catalytic activity in serine proteases. Proc. Natl Acad. Sci. USA **93:** 10653–10656
29 Guinto E. R., Vindigni A., Ayala Y. M., Dang Q. D. and Di Cera E. (1995) Identification of residues linked to the slow-fast transition of thrombin. Proc. Natl Acad. Sci. USA **92:** 11185–11189
30 Wu Q., Sheehan J. P., Tsiang M., Lentz S. R., Birktoft J. J. and Sadler J. E. (1991) Single amino acid substitutions dissociate fibrinogen-clotting and thrombomodulin-binding activities of human thrombin. Proc. Natl Acad. Sci. USA **88:** 6775–6779
31 Gibbs C. S., Coutre S. E., Tsiang M., Li W. X., Jain A. K., Dunn K. E. et al. (1995) Conversion of thrombin into an anticoagulant by protein engineering. Nature **378:** 413–416
32 Tsiang M., Paborsky L. R., Li W. X., Jain A. K., Mao C. T. et al. (1996) Protein engineering thrombin for optimal specificity and potency of anticoagulant activity in vivo. Biochemistry **35:** 16449–16457
33 Hanson S., Harker L., Kelly A., Fernandez J., Griffin J. and Gibbs C. S. (1997) Antithrombotic effects of selective activation of endogenous protein C. Thromb Haemost (June) Suppl: 419
34 Berg D. T., Wiley M. R. and Grinnell B. W. (1996) Enhanced protein C activation and inhibition of fibrinogen cleavage by a thrombin modulator. Science **273:** 1389–1391
35 Rezaie A. R. (1996) Tryptophan 60-D in the B-insertion loop of thrombin modulates the thrombin-antithrombin reaction. Biochemistry **35:** 1918–1924
36 Tsiang M., Jain A. K. and Gibbs C. S. (1997) Functional requirements for inhibition of thrombin by antithrombin III in the presence and absence of heparin. J. Biol. Chem. **272:** 12024–12029