Thrombin Interactions*

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After generation from prothrombin, thrombin plays multiple roles in the blood coagulation cascade that are mediated by interaction with a number of physiologic substrates, effectors, and inhibitors. Structural and mutagenesis studies have helped unravel the molecular basis of thrombin interactions in the context of both well-established and emerging new roles of the enzyme. The functional versatility of thrombin owes much to its evolutionary origin and results from structural determinants and mechanisms that can be exploited by pharmacologic intervention. ***(CHEST 2003; 124:11S–17S)***

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Abbreviation: PAR = protease-activated receptor

Blood coagulation evolved as a specialization of the complement system and immune response,[1] which in turn bear close evolutionary ties with developmental enzyme cascades.[2] Indeed, the physiologic response to vascular injury in vertebrates is the product of molecular mechanisms that first emerged in extracellular matrix reorganization, cell signaling, and host defense. The functional sophistication of thrombin, the key enzyme of the coagulation cascade, is best understood in the context of its evolutionary origin.

Thrombin diverged from the complement factors C1r, C1s, or MASP-2, heralding the onset of further specialization of defense mechanisms in the deuterostome lineage.[1,2] The ancestral link between clotting and immunity is reinforced by the observation that sequence homologues of fibrinogen, the terminal substrate in blood clotting and a specific substrate of thrombin, originally served in immunologic roles.[3] Thrombin predated and most likely gave rise to all other vitamin K-dependent proteases,[1] namely factors VIIa, IXa, and Xa, which define the convergence between the intrinsic and extrinsic pathways of the coagulation cascade, and activated protein C, which adds negative regulation to the cascade and a link to the universe of reactions defining the inflammatory response.[4] Because complement evolved from developmental proteases,[1,2] as also documented by the dual role played by the Toll signaling pathway in development and host defense,[5] it is not surprising that thrombin itself retains signatures of its descent from a growth factor. In the mouse, knockout of the prothrombin gene results in embryonic and neonatal lethality,[6,7] a wastage not seen with mouse models deficient for platelets[8] or fibrinogen,[9] and that therefore does not depend on thrombin as a coagulation factor. Thrombin is expressed not only in the liver, the major site of clotting factor synthesis, but also in the developing and adult rat brains.[10] Thrombin activates protease-activated receptors (PARs), a small but important subgroup of the G protein-coupled receptor superfamily (see the article by Dr. Brass in this supplement),[8] promoting platelet aggregation as well as tumor growth and metastasis,[11] angiogenesis (see the article by Dr. Rickles in this supplement),[12] atherosclerosis and inflammation,[13] the survival or apoptosis of glial cells and neurons,[14] myoblast survival,[15] and neutrophil chemotaxis.[16] Thrombin also acts nonproteolytically to induce monocyte chemotaxis,[17] and prothrombin can promote the migration of cells through the extracellular matrix,[18] an activity crucial for both embryonic development and tumor metastasis.

The plethora of biological functions in which thrombin plays a dominant role is a testimony to a long and rich evolutionary history. The molecular basis of this functional complexity has now become accessible through detailed investigations of the structure and enzymatic properties of the enzyme.

Thrombin Substrates

After its generation from prothrombin (see the article by Dr. Mann in this supplement),[19] thrombin is engaged in paradoxically opposing functions in the blood.[20] As a procoagulant factor, thrombin converts fibrinogen into an insoluble fibrin clot that anchors platelets to the site of lesion and initiates processes of wound repair. This action is reinforced and amplified by activation of the transglutaminase factor XIII that covalently stabilizes the fibrin clot, the inhibition of fibrinolysis (see the article by Dr. Nesheim in this supplement), and the proteolytic activation of factors V, VIII, and XI. In addition, thrombin acts as an anticoagulant when it activates protein C (see the article by Dr. Esmon in this supplement). This function unfolds on binding to thrombomodulin, a receptor on the membrane of endothelial cells. Binding of thrombomodulin suppresses the ability of thrombin to cleave fibrinogen, but enhances $>$ 1,000-fold the specificity of the enzyme toward the zymogen protein C. The reaction is assisted by the presence of a specific endothelial cell protein C receptor.[21] Activated protein C cleaves and inactivates factors Va and VIIIa, two essential cofactors of coagulation factors Xa and IXa that are required for thrombin generation, thereby down-regulating both the amplification and progression of the coagulation cascade. Activated protein C also acts as an immediate-response anti-inflammatory enzyme through cellular signaling and links blood coagulation to inflammation.[4,13] Scavenging of thrombin by thrombomodulin and activation of protein C in the microcirculation constitute an important pathway of regulation of the coagulation response.[22] Additional regulation is achieved when thrombin is irreversibly inhibited at the

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active site by the stoichiometric serine protease inhibitor (serpin) antithrombin with the assistance of heparin.[23] Heparin provides a template for optimal docking of antithrombin onto the thrombin active site and accelerates the rate of inactivation $> 1,000$-fold. Thrombin can also be inactivated stoichiometrically by the serpin heparin cofactor II using glycosaminoglycans as cofactors.

Other roles of thrombin unfold on cleavage and activation of PARs. Four PARs have been cloned,[24,25] and all share the same basic mechanism of activation: thrombin and other proteases, derived from the circulation and inflammatory cells, cleave at a specific site within the extracellular N-terminus to expose a new N-terminal tethered ligand domain, which binds to and activates the cleaved receptor.[8] Thrombin activates PAR-1,[26] PAR-3,[27] and PAR-4[28,29] in this manner. Activation of PAR-1 and PAR-4 on human platelets triggers platelet activation and aggregation and unfolds the prothrombotic role of thrombin in the blood.[8,30] PAR-3 is not present on human platelets, but is widely and abundantly expressed in other cell types.[24]

Substrate Recognition

The wealth of thrombin interactions presages a rich repertoire of mechanisms underlying substrate recognition. Valuable insights into such mechanisms are garnered from considering the rate at which thrombin interacts with substrate to form a productive complex that can proceed to catalysis, as well as the energy barrier associated with this process. Highly specific interactions are characterized by modest energetic barriers (5 to 10 kcal/mol) and fast rates of complex formation, approaching the diffusion-controlled limit (6×10^8 $M^{-1}s^{-1}$).[31] The thrombin interactions responsible for the procoagulant (fibrinogen and fibrin cleavage) and prothrombotic/signaling (cleavage of the PARs) functions carry signatures of high specificity (Figs 1, 2). Fibrinogen, fibrin, and PAR-1 bind rapidly to thrombin with rates approaching the theoretical limit of diffusion control. The energy barrier for the interaction is small, which suggests a high degree of complementarity between enzyme and substrate. In contrast, the anticoagulant activity of thrombin (cleavage of protein C) is characterized by a significantly lower rate of productive complex formation and a very high energy barrier. This underscores the presence of significant conformational rearrangements on formation of the complex, most likely of the induced-fit type. The presence of thrombomodulin accelerates the rate at which thrombin and protein C come together, but does not relieve the energetic cost of the interaction. This is typical of template-based interactions where the cofactor (thrombomodulin) provides a scaffold for the enzyme (thrombin) and substrate (protein C) to optimize the chances of a productive collision. A similar mechanism can be invoked for the thrombomodulin-induced enhancement of thrombin interaction with thrombin-activated fibrinolysis inhibitor[32] and explains the heparin-induced enhancement of thrombin inactivation by antithrombin.[23]

Thrombin Structure: Role of the Exosites

Thrombin is composed of two polypeptide chains, A and B, that are covalently linked through a disulfide bond. The A chain (36 residues) has no documented functional role and runs opposite to the front hemisphere of the B chain (259 residues), which hosts the entrance to the active site and all functional epitopes of the enzyme. The B chain is shaped like a sponge, with deep crevices and large protuberances on its water-accessible surface (Fig 3).[33] The active-site cleft appears as an invagination at the center of the molecule. Thrombin features a trypsin-like specificity and cuts preferentially at Arg residues of substrate. Unlike trypsin, however, thrombin cleaves selectively at specific Arg sites using ancillary interactions from "exosites" distinct from the active site.

Exosite I is located "east" of the active site (Fig 3). It contains hydrophobic patches and numerous charged res-

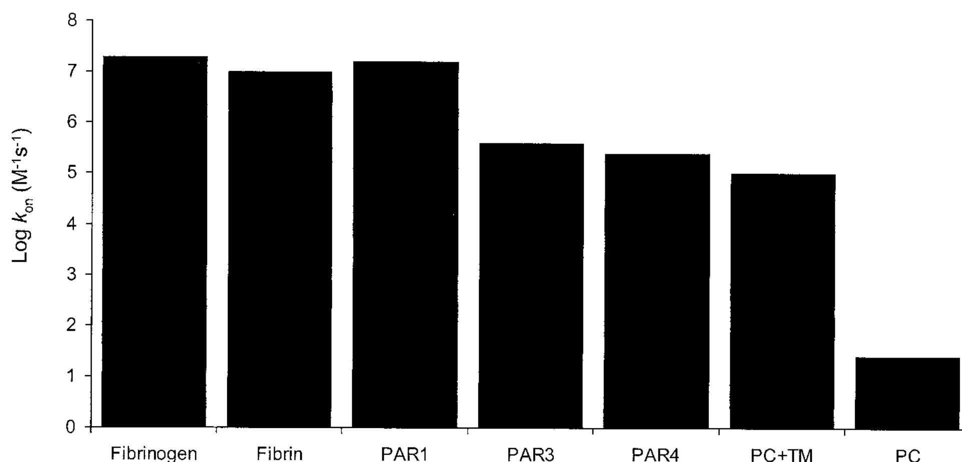

Figure 1. Rate of productive complex formation, *k*on, (log scale) for several thrombin substrates. The procoagulant (fibrinogen, fibrin) and prothrombotic (PAR-1, PAR-4) substrates bind to thrombin significantly faster than the anticoagulant substrate protein C (PC) with or without thrombomodulin (TM) present.

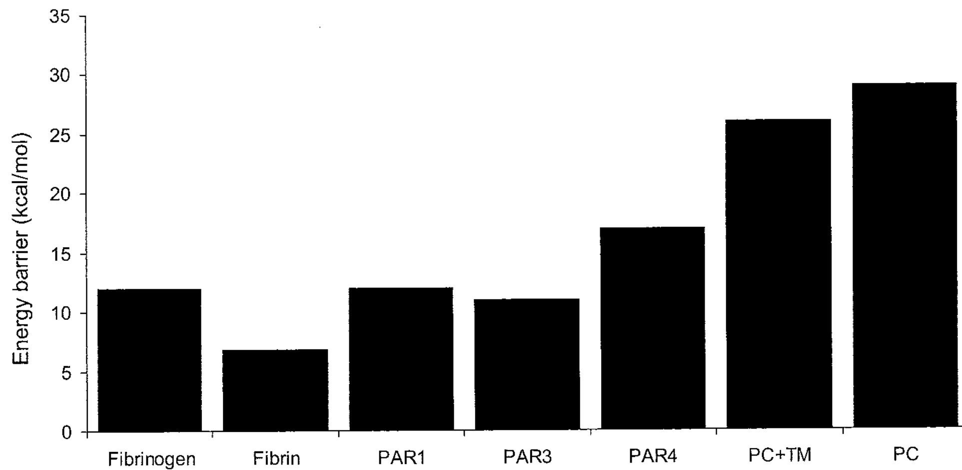

FIGURE 2. Energy barrier associated with formation of the thrombin-substrate complex. The procoagulant (fibrinogen, fibrin) and prothrombotic (PAR-1, PAR-4) substrates bind to thrombin by overcoming a significantly smaller energy barrier compared with protein C. Thrombin is optimized for interaction with fibrinogen, fibrin, and the PARs, but requires induced-fit rearrangements to interact with protein C. See Figure 1 legend for expansion of abbreviations.

idues on its surface that provide electrostatic steering to fibrinogen on its approach to the active site of thrombin. This facilitated diffusion accounts for the fast rate of complex formation (Fig 1). Fibrinogen circulates in the plasma as a dimer of three chains, $(A\alpha B\beta\gamma)_2$, that are covalently linked by disulfide bonds.[34] Thrombin attacks the Aα chain first to release fibrinopeptide A and to generate fibrin I monomer. Fibrin then assembles into protofibrils that are attacked by thrombin at the Bβ chains, leading to the release of fibrinopeptide B and the assembly of thick fibers forming the scaffold of the ensuing clot. Fibrinogen requires the integrity of exosite I for docking onto the thrombin surface, and its epitope spans from exosite I to the active site (Fig 4).[35] A similar epitope is used by thrombin to interact with the potent natural inhibitor hirudin,[36] PAR-1,[37] and fibrin.[38] Therefore, the procoagulant and prothrombotic/signaling functions of thrombin are closely intertwined and difficult to dissociate. However, there is incomplete overlap among the epitopes recognizing PAR-1, PAR-3, and PAR-4.[38] Residues important for PAR-4 binding cluster around the access to the active site. In the case of PAR-3, the situation is intermediate to PAR-1 and PAR-4. The region around the active site follows the hierarchy of importance in recognition PAR-4 > PAR-3 > PAR-1, whereas exosite I follows the hierarchy PAR-1 > PAR-3 > PAR-4.

Exosite I also provides the locale for the binding of thrombomodulin to thrombin.[39] Human thrombomodulin contains five distinct domains: an N-terminal lectin-like domain, six epidermal growth factor domains, a glycosylated Ser/Thr-rich domain, a transmembrane domain, and a C-terminal cytosolic tail. The epidermal growth factor-like domains 5 and 6 are essential for thrombomodulin binding to thrombin via exosite I.[40] The epitope of thrombin recognizing thrombomodulin in exosite I overlaps completely with those of fibrinogen, fibrin, and PAR-1 (Fig 4). Because of this overlap, thrombomodulin binding abrogates the procoagulant and prothrombotic activities of thrombin. At the same time, thrombomodulin promotes cleavage of the anticoagulant protein C by thrombin by providing a template for the rapid formation of the enzyme-substrate complex (Fig 1). Protein C contacts the thrombin surface away from exosite I and explores marginally the areas involved in fibrinogen and PAR-1 recognition (Fig 4).[41] Thrombin-activated fibrinolysis inhibitor utilizes a similar epitope for binding to thrombin,[42] as does antithrombin in the presence of heparin.[43] The dissociation of the epitopes recognizing protein C and fibrinogen is striking and has important practical implications (see below).

Exosite II is positioned "west" of the active site (Fig 3), opposite to exosite I. It features a conspicuous number of

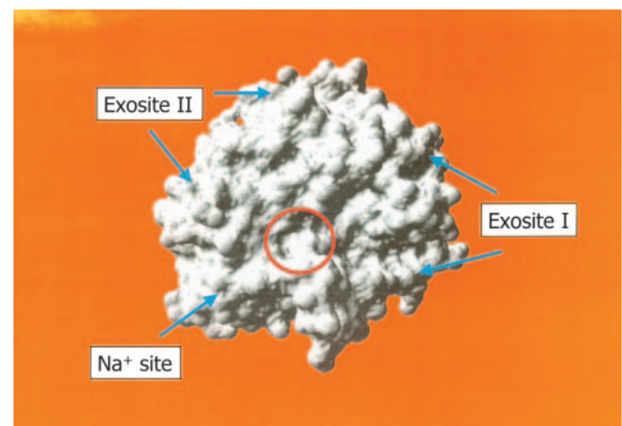

FIGURE 3. Schematic representation of the water-accessible surface of the B chain of thrombin.[33] The active-site region is circled. Note the location of exosite I (east), exosite II (west), and the Na^+ binding site (southwest) relative to the active site.

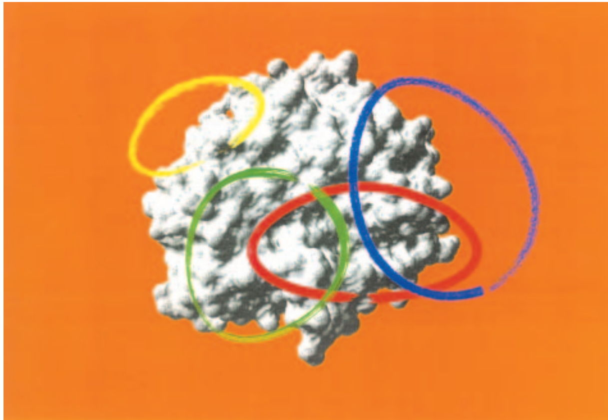

FIGURE 4. Schematic representation of the water-accessible surface of the B chain of thrombin,[33] as shown in Figure 3. The epitopes recognizing substrates and effectors are depicted schematically by ovals. Fibrinogen and fibrin bind to thrombin by occupying exosite I and the active-site region (red oval). This binding mode is also shared by PAR-1, PAR-3, and the natural inhibitor hirudin. Thrombomodulin binds exclusively to exosite I (blue oval), overlapping with the epitopes recognizing fibrinogen and PAR-1 in this region. This allows thrombomodulin to completely abrogate the procoagulant and prothrombotic activities of thrombin. Protein C binds around the Na^+ site and the active-site region (green oval), overlapping minimally with fibrinogen and PAR-1 binding. This binding mode is also observed for antithrombin III and TAFI. Heparin binds to exosite II (yellow oval). The complete overlap between the epitopes recognizing fibrinogen and PAR-1 makes dissociation of the procoagulant and prothrombotic activities of thrombin difficult. However, the incomplete overlap between the epitopes recognizing protein C and fibrinogen makes it possible to engineer anticoagulant thrombin mutants with selective activity toward protein C.

charged residues, but unlike exosite I has no hydrophobic patches on its surface. Exosite II is the locale for interaction with polyanionic ligands like glycosaminoglycans and heparin.[44] Heparin is a potent anticoagulant owing to its role as a cofactor of antithrombin, the serpin that specifically and irreversibly shuts down the activity of thrombin and coagulation factor Xa.[23] The importance of this inhibitory interaction is demonstrated by the increased incidence of thrombosis in individuals with acquired or inherited deficiencies of antithrombin,[45] and the embryonic lethality of the knockout in the mouse.[46] The structural determinant required for antithrombin binding is a unique pentasaccharide sequence that is randomly distributed along the heparin chains. This determinant is required of both unfractionated heparin, a mixture of polysaccharide chains in the molecular weight range of 3,000 to 30,000 d, and low-molecular-weight heparins, with a molecular weight of approximately 5,000 d. Any pentasaccharide containing heparin can bind and activate antithrombin. However, while this activation is sufficient to inhibit factor Xa, a specific bridge-binding interaction of heparin with antithrombin and thrombin itself is necessary to inactivate this enzyme. The ternary complex can only form with pentasaccharide containing heparin chains of at least 18 saccharide units and enhances $>$ 1,000-fold the rate of thrombin inactivation. Interestingly, the specificity of the interaction is not stored in the reactive center loop of antithrombin, but in separate exosite regions that secure the inhibitor to the thrombin or factor Xa surface.[23] Through this strategy, antithrombin cleverly avoids inhibition of activated protein C, which shares with thrombin and factor Xa several determinants of active-site specificity, and ensures the very existence of the protein C pathway.[23] Glycosaminoglycans facilitate the inactivation of thrombin by the serpin heparin cofactor II. This assistance, however, is not as crucial as for antithrombin because heparin cofactor II utilizes an ancillary interaction with exosite I via its acidic N-terminal domain,[47] thereby engaging thrombin in a double bridge of exosites I and II.

Thrombin Is an Allosteric Enzyme

An important mechanism of regulation of thrombin function unfolds on binding of Na^+ between two loops located “southwest” of the active site (Fig 3).[48] Binding of Na^+ converts thrombin from the slow (Na^+-free) to the fast (Na^+-bound) form, and enhances substrate binding and catalysis. The effect is exquisitely allosteric because the Na^+ binding site is located away from residues of the catalytic machinery in the active site. The slow and fast forms are significantly populated (2:3 ratio) under physiologic conditions because the Na^+ concentration in the blood (140 mmol/L) is not sufficient to saturate the site. Hence, the slow $\leftrightarrow$ fast equilibrium *in vivo* is optimally poised for allosteric regulation, which is all the more significant because the procoagulant and anticoagulant activities of thrombin are partitioned between the fast and slow forms, respectively.[49] The fast form has higher specificity than the slow form for fibrinogen, fibrin, PAR-1, PAR-3, and PAR-4, whereas the slow form is slightly more specific than the fast form for the anticoagulant substrate protein C. Na^+ binding enhances significantly ($>$ 20-fold) the procoagulant (cleavage of fibrinogen) and prothrombotic (cleavage of PAR-1 and PAR-4) activities of thrombin, but has no effect on the anticoagulant (cleavage of protein C) activity of the enzyme (Fig 5). The procoagulant role of the fast form is further supported by its prominence in the activation of factor V[50] and factor VIII.[51] However, antithrombin preferentially inhibits the fast form, thereby indirectly boosting the anticoagulant potential of the slow form.

Due to the allosteric nature of thrombin, any effect that destabilizes Na^+ binding stabilizes the slow form and produces an anticoagulant effect by prolonging the clotting time (reduced fibrinogen cleavage) and reducing platelet activation (reduced PAR-1 and PAR-4 cleavage). Naturally occurring mutations of residues involved in Na^+ binding, as seen in prothrombin Greenville[52] and prothrombin Scranton,[53] are associated with bleeding phenotypes. Among the known allosteric effectors of thrombin, fibrin shifts the equilibrium in favor of the fast form and promotes fibrinogen clotting. Heparin, however, has the opposite effect and stabilizes the slow form. Na^+ itself can become the arbiter of the procoagulant/anticoagulant fate of thrombin. The Na^+ concentration in the blood is tightly controlled, but hyponatremia ($Na^+ < 135$ mEq/L)[54] and hypernatremia ($Na^+ > 145$ mEq/L)[55] are among the most

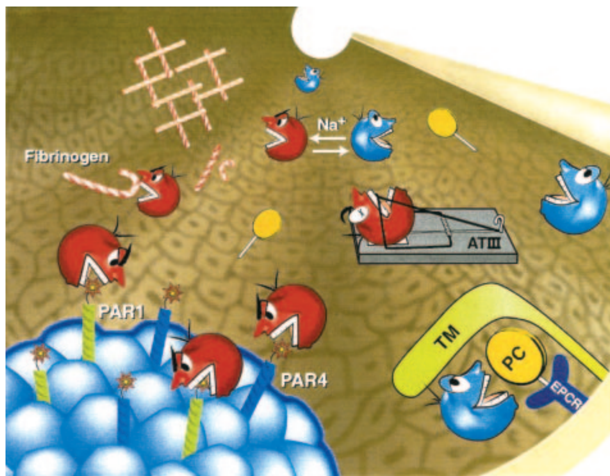

FIGURE 5. Schematic representation of the roles of the Na^+-free slow (blue) and Na^+-bound fast (red) forms of thrombin in blood coagulation. The two forms are in rapid equilibrium, producing a 2:3 ratio distribution in favor of the fast form under physiologic Na^+ concentrations (140 mmol/L). The fast form has procoagulant and prothrombotic activities. It cleaves fibrinogen, PAR-1, and PAR-4 with high specificity. This form is also inhibited very effectively by antithrombin (ATIII). The slow form, however, is anticoagulant. It has low specificity toward fibrinogen, PAR-1, and PAR-4, but retains high specificity toward protein C, which is cleaved with the assistance of thrombomodulin and the endothelial protein C receptor (EPCR). Any effect that alters the equilibrium between the slow and fast forms of thrombin has a profound impact on the procoagulant/anticoagulant fate of this enzyme. Factors that stabilize thrombin in the fast form (hypernatremia, fibrin) enhance fibrinogen clotting and platelet aggregation and promote thrombosis. Factors that stabilize the slow form (hyponatremia, heparin, naturally occurring mutations of prothrombin in the Na^+-binding site) impair fibrinogen clotting and platelet aggregation and promote bleeding. See Figure 1 legend for expansion of abbreviations.

common electrolyte disorders encountered by primary care providers, nephrologists, and pediatricians. Hypernatremia is often associated with venous thrombosis, especially of the cerebral vasculature[56] or of the lower extremities if secondary to diabetes.[55] Infusion of hypertonic saline solution in healthy volunteers results in increased levels of fibrinopeptide A, a product of fibrinogen cleavage by thrombin, and a significant reduction in its generation time.[57] The association of bleeding due to reduced clotting secondary to hyponatremia is more difficult to document, because one of the major causes of hyponatremia is subarachnoid hemorrhage.[54] However, some independent evidence of increased bleeding secondary to hyponatremia has been reported in infants.[58]

The thrombotic tendency induced by increases in the plasma levels of Na^+ has much to do with stabilization of thrombin in the procoagulant fast form. Reinforcing the critical procoagulant role of Na^+ in the coagulation cascade is the discovery of Na^+-dependent allosteric effects in other clotting enzymes, like factor Xa,[59] factor VIIa,[60] and activated protein C.[61] These enzymes possess a Na^+ binding site homologous to the one first identified in thrombin. Indeed, Na^+ binding to factor Xa enhances the catalytic activity of the prothrombinase complex responsible for prothrombin activation,[59] an effect that couples synergistically to the Na^+-induced enhancement of fibrinogen cleavage by thrombin.

Thrombin and Anticoagulant Therapy

The study of thrombin interactions has enriched our understanding of clotting enzymes in general and has been instrumental to the development of new anticoagulants. Anticoagulant therapy is currently dominated by heparinoids, which assist antithrombin in the inhibition of thrombin and factor Xa, and warfarin, a generic drug that inhibits the synthesis of all vitamin K-dependent factors (see the article by Dr. Heit in this supplement).[62] Unfractionated heparin and warfarin are beset by a narrow therapeutic window and an almost unpredictable dose-response profile. Other limitations of unfractionated heparin are the potential to trigger heparin-induced thrombocytopenia and the inability to promote inhibition of fibrin-bound thrombin and platelet-bound factor Xa. Low-molecular-weight heparins feature a more predictable dose-response relation compared with the unfractionated forms and are devoid of the former limitation, but not the latter.[63] Direct inhibitors of thrombin have long captivated the interest of medicinal chemists in the hope that they could feature a broader therapeutic window compared with heparin and warfarin and afford an efficient inhibition of thrombin free or bound to the fibrin clot. Following the development of argatroban as a potent active site inhibitor, the thrombin inhibitor bivalirudin was designed rationally from the results of studies on how thrombin recognizes fibrinogen and the potent natural inhibitor hirudin. Hirudin and bivalirudin are highly selective thrombin inhibitors because, unlike argatroban, which only binds to the active site, they exploit a large surface of recognition that spans from the active site to exosite I. However, the current direct thrombin inhibitors do not have a superior therapeutic efficacy and safety compared with low-molecular-weight or even unfractionated heparins.[63]

Remarkably, both argatroban and bivalirudin were developed before the first structure of thrombin became available.[33] With > 150 structures of thrombin currently deposited in the Protein Data Bank, there is enough incentive to explore new avenues in the design of the next generation of direct thrombin inhibitors. The success of bivalirudin, which bridge binds to exosite I and the active site, mimicking the natural inhibitor hirudin and the binding mode of fibrinogen and PAR-1, should draw attention to alternative inhibitors, like hemadin,[64] that exploit bridge binding to exosite II and the active site. Even more compelling would be the design of allosteric inhibitors of thrombin that act on the Na^+ binding site, which should now be made possible by the recently solved structure of the anticoagulant slow form.[65]

Thrombin itself should be given consideration as an anticoagulant when selectively deprived of its ability to interact with fibrinogen and PAR-1. Following pioneering observations on the direct anticoagulant potential of low-dosage thrombin administration in baboons,[66] thrombin mutants with selective specificity toward the anticoagulant

protein C have been rationally engineered and show potent and safe anticoagulant and antithrombotic effects *in vivo*.[67,68] One of these mutants is more potent and safer than the administration of activated protein C itself,[68] which raises important questions about how the treatment of thrombotic disorders and complications may benefit from administration of activated protein C.[4,13]

Concluding Remarks

The wealth of structural and functional information garnered on the biological roles of thrombin is substantial. New paradigms emerging from the structure and allosteric regulation of thrombin have enlightened our growing understanding of the mechanisms of substrate recognition in this and related clotting enzymes. This new knowledge defines untapped opportunities for the pharmacologic control of blood coagulation and will secure thrombin a primary role among clotting proteases for years to come.

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