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Exosites in the substrate specificity of blood coagulation reactions

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Summary

The specificity of blood coagulation proteinases for substrate, inhibitor, and effector recognition is mediated by exosites on the surfaces of the catalytic domains, physically separated from the catalytic site. Some thrombin ligands bind specifically to either exosite I or II, while others engage both exosites. The involvement of different, overlapping constellations of exosite residues enables binding of structurally diverse ligands. The flexibility of the thrombin structure is central to the mechanism of complex formation and the specificity of exosite interactions. Encounter complex formation is driven by electrostatic ligand–exosite interactions, followed by conformational rearrangement to a stable complex. Exosites on some zymogens are in low affinity proexosite states and are expressed concomitant with catalytic site activation. The requirement for exosite expression controls the specificity of assembly of catalytic complexes on the coagulation pathway, such as the membrane-bound factor Xa•factor Va (prothrombinase) complex, and prevents premature assembly. Substrate recognition by prothrombinase involves a two-step mechanism with initial docking of prothrombin to exosites, followed by a conformational change to engage the FXa catalytic site. Prothrombin and its activation intermediates bind prothrombinase in two alternative conformations determined by the zymogen to proteinase transition that are hypothesized to involve prothrombin (pro)exosite I interactions with FVa, which underpin the sequential activation pathway. The role of exosites as the major source of substrate specificity has stimulated development of exosite-targeted anticoagulants for treatment of thrombosis.

Keywords

blood coagulation; exosites; factor Va; factor Xa; prothrombin; serine proteinases; zymogens

Introduction

Blood coagulation proteinases have evolved versatile and sensitive regulatory mechanisms for controlling the specificity of protein substrate recognition mediated by exosites. Exosites are surface sites on proteinases physically separated from the active site residues that determine the primary S1–S4¹ peptide substrate and inhibitor specificity. The concept of exosites in coagulation proteinases was introduced by John Fenton et al. around 1977 [2], and in molecular modeling of coagulation proteinase domains by Furie, Bing *et al.* in 1982 [3]. An extended substrate recognition site on thrombin, proposed to be intimately involved in its specificity for cleaving fibrinogen [4], was subsequently called the fibrinogen recognition exosite, or anion-

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¹Schechter-Berger [1] notation referring to the residues of a substrate (from the N-terminal end) as ...P4-P3-P2-P1-P1'-P2'... with the scissile bond at P1-P1'. The corresponding specificity subsites on the proteinase are designated...S4-S3-S2-S1-S1'-S2'...

binding exosite I due to its apparent affinity for negatively charged ligands [4]. Molecular modeling of the catalytic domains of thrombin, factor (F) Xa, and FIXa with chymotrypsin as a template, led to the prescient conclusion that substrate specificity is not determined primarily by the active site, but by surface insertion loops absent in chymotrypsin and unique to each coagulation proteinase, surrounding their catalytic sites [3]. After about 30 years of intensive research and numerous crystal structures of thrombin and its exosite ligand complexes, thrombin has emerged as the prototypical example of an exosite-regulated allosteric proteinase.

Many excellent reviews of the roles of exosite interactions in coagulation proteinase substrate specificity have been published previously, including many focused on the best studied thrombin exosites [5–11]. This review will focus on less covered aspects, such as: (i) structural and functional properties of thrombin exosites with a consideration of the dynamics of exosite-mediated protein–protein interactions; (ii) the role of low affinity exosite precursor forms (proexosites) and their expression via the zymogen–proteinase transition in controlling blood coagulation substrate specificity, in the context of prothrombin (ProT) activation; (iii) regulation of the substrate specificity of membrane-assembled proteinase–protein cofactor complexes, for the mechanism of substrate recognition by the prothrombinase complex; (iv) the role of FXa and (pro)thrombin exosites in kinetic control of the ProT activation pathway; and (v) the potential of developing anticoagulant therapy by targeting exosites.

Thrombin exosites

Two electropositive exosites, in near opposition on the thrombin surface, play crucial roles in the recognition of specific macromolecular substrates, effectors, and inhibitors, and their properties have been characterized by extensive crystallographic, mutagenesis, biophysics, and enzymology studies. Exosite I, composed of insertion loops 30–40 and 70–80 [12,13], mediates binding of fibrinogen, fibrin, PAR-1 and -4 substrate recognition, staphylocoagulase binding, and binding of the C-terminal 55–65 residue sequence of the thrombin-specific inhibitor, hirudin [5–8,11,14,15]. Exosite II, a positively charged cluster of residues, mediates different interactions specifically, including heparin [16], a specific monoclonal antibody [17], and the fragment 2 domain of ProT (F2) [18]. A variety of inhibitors from snake venoms and the saliva of hematophagous organisms also bind to thrombin exosites [11,19].

The thrombin catalytic site and the affinity of exosite I ligands are allosterically regulated by binding of Na⁺ [9,20,21]. Na⁺ binding switches thrombin from a ‘slow’ to a ‘fast form’ with higher specificity (k_{cat}/K_m) for the procoagulant substrates, fibrinogen, and PAR-1, greater reactivity toward antithrombin, and increased peptide substrate activity [8,20,22]. The Na⁺-free, slow form has higher relative specificity in protein C activation [22]. Thus, Na⁺ regulates the procoagulant and anticoagulant activities of thrombin. The structure of the slow form has been controversial, in that inactive thrombin conformations have also been concluded to represent the slow form [23–25]. Crystal packing interactions that influenced interpretation of previous structures of the fast, slow, and inactive forms are absent from the structure of thrombin D102N, which is thought to represent more closely the inactive slow form [26]. The thrombin D102N structure, and kinetic studies of Na⁺ binding [26–28], indicate that the catalytically active slow form is in equilibrium with a low level of inactive thrombin in which the catalytic and Na⁺ sites are blocked. Rapid-reaction kinetic studies of Na⁺ binding indicate that at 37 °C the inactive slow form is insignificantly populated [28]. Structures of the fast, slow, and inactive forms demonstrate dramatically the flexibility of thrombin.

Alanine scanning studies of thrombin exosite complexes provide a basis for interpreting the mechanisms involved in specific binding of structurally diverse protein substrates with strict specificities for exosite I or II. The structures illustrate a mechanism of specific ligand recognition in which the conformational flexibility of thrombin is necessary to accommodate

diverse ligands with high specificity for each exosite. Figure 1 illustrates the structure of thrombin with exosite I residues that are most important for binding of particular ligands. It is apparent that exosite I-specific ligands bind competitively through overlapping constellations of distinctly different residues critical to each interaction. The epitope for Y63-sulfated hirudin-(55–65) involves exosite I extensively [15,29]. Fibrinogen [30,31] and PAR-1 [32] binding to exosite I overlap with the hirudin peptide and include many of the same residues. The extensive overlap in exosite I epitopes accounts for competitive binding of the hirudin peptide, fibrinogen, and PAR-1.

Rapid-reaction kinetic studies of hirudin and hirudin-(54–65) binding to thrombin support electrostatic steering of anionic exosite I ligands, such as the peptide, which mediates initial weak encounter complex formation, followed by establishing direct ionic interactions, and final conformational rearrangement of the exosite and ligand by engagement of hydrophobic interactions [33–35]. Thermodynamic studies of thrombin–hirudin interactions also support significant changes in the 70–80 loop of exosite I and structural ordering of the peptide on complex formation [36,37]. It has been suggested that all exosite I ligands follow a similar model, in which complementary asymmetric electrostatic fields enhance the rate of complex formation [35]. In agreement with this idea, analysis of the ionic strength dependence of the kinetics of fibrinogen cleavage by thrombin similarly supports diffusion-controlled electrostatic steering that facilitates initial formation of the transition state [38].

Exosite II interactions are stabilized mainly by electrostatic interactions, whereas exosite I interactions involve a greater contribution from hydrophobic interactions [6]. Exosite II-specific interactions with heparin [16] and ProT F2 [39] overlap to some extent (Fig. 2), but with only one common residue. As part of the GpIb-IX-V platelet receptor complex, GpIb α acts as a thrombin cofactor, producing a 5- to 7-fold enhancement in PAR-1 activation and 6- to tenfold acceleration of GpV cleavage, resulting in platelet activation, and a five thousandfold enhancement of FXI activation [40]. Two divergent crystal structures of GpIb α –thrombin complexes showed two thrombin molecules bound, one through exosite I and the other exosite II [41,42], which continued the controversy concerning the mode of thrombin binding. Mutagenesis studies with eight alanine substitutions of exosite II residues and two charge reversal mutants demonstrated decreased thrombin affinity [43,44], indicating exosite II binding (Fig. 2), whereas more limited exosite I mutagenesis showed no effect on GpIb α binding [43–45].

Some interactions make use of both exosites in determining binding specificity, presumably enhancing affinity and providing more strict control over the orientation of the bound thrombin for specific cleavages (Fig. 3). Binding of individual ligands to each exosite is typically accompanied by changes in tripeptide substrate specificity, and distinct fluorescence spectral changes reporting each binary interaction with thrombin active site-specifically labeled with tripeptide chloromethyl ketone-tethered fluorescence probes [46–50]. It had been suggested that exosite I and II binding was subject to extremely negative thermodynamic linkage, such that simultaneous interactions with both exosites was prevented allosterically [51]. An evaluation of linkage between binding of the exosite I ligands, fibrinogen, and hirudin peptide, and exosite II binding of a monoclonal antibody and F2 demonstrated no detectable inter-exosite linkage [17,50]. These studies concluded instead that binding of ligands to each exosite is linked to changes in the environment of the catalytic site. Ternary complexes formed with non-interacting ligands for both exosites produce additive or non-additive fluorescence changes, reporting changes in the active site environment [48,50]. These studies illustrate the sensitive linkage between the thrombin active site and exosite binding.

Thrombomodulin interacts with exosite I residues [30,52] that also overlap those involving the hirudin peptide [29], fibrinogen [30], and PAR-1 binding [32], and with exosite II via its

chondroitin sulfate moiety (Fig. 3). Factor V [53] and FVIII [54] substrate recognition are mediated primarily by a distinct group of exosite I residues, which are subsets of those involving the hirudin peptide. The epitopes overlap, but a smaller cluster of residues contributes to FVIII activation. Three important exosite II residues are involved in FV activation, while mutation of only one residue in exosite II has significant effects on FVIII activation. The differences between these interactions explain the primary involvement of exosite I and the secondary role of exosite II in FV and VIII activation [47,55–57]. Alanine scanning of heparin cofactor II (HCII) inactivation of thrombin showed interactions with exosite I to be important in the reaction, while only one residue in exosite II could be implicated in heparin acceleration [58].

The observations concerning the dynamics and electrostatic steering of thrombin protein–protein interactions parallel recent studies of the roles of flexibility and overlapping sites in the general mechanisms of protein–protein interactions. A good example is the binding of Enzyme I to the phosphocarrier protein of a bacterial signaling system [59,60]. Intermolecular paramagnetic relaxation enhancement studies using paramagnetic probe-labeled phosphocarrier protein were performed to detect sparsely populated complexes. Molecular modeling was used to generate a probability distribution structure of the non-specific encounter complexes. Many structurally distinct alternative encounter complexes are formed at low concentrations initially through electrostatic interactions. This is coupled to intramolecular rearrangement and water desolvation to the most stable complex observed crystallographically. This electrostatic searching mechanism of protein–protein interactions undoubtedly plays a major role in regulating the sensitivity and specificity of coagulation proteinase exosite binding. Molecular dynamics studies of protein–protein interactions also support a similar general model based on the critical role of flexibility [61].

Other experimental approaches have extended the understanding of the extent of thrombin conformational plasticity. Mapping mobility of thrombin by amide hydrogen/deuterium exchange demonstrated decreases in exchange of exosite I peptides accompanying active site inactivation with *D*-FPR-CH₂Cl [62]. Comparison of ProT and thrombin showed that exosite I was more flexible in thrombin, whereas the autolysis loop and Na⁺ binding site exchanged more slowly [63]. Binding of a peptide from the γ'-fibrinogen variant to exosite II demonstrated that the 70–80 loop of exosite I and the autolysis loop were slower exchanging in the complex, suggesting long-range exosite I and II communication [64]. The source of the changes in intrinsic protein fluorescence accompanying binding of Na⁺ has been mapped by Phe substitution of each of the nine Trp residues [28]. Surprisingly, perturbation of all Trp residues occurred with formation of the thrombin•Na⁺ complex, with the largest contributions from W215 and W141, but significant, non-additive increases or decreases in fluorescence for all other Trp residues. The fluorescence and hydrogen/deuterium exchange results may reflect low energy structural rearrangements that may not be revealed by loss of function mutagenesis. W215 and W141 made equivalent, dominant contributions to the fluorescence change, but substitution of these or any of the other Trp residues was not associated with a significant change in Na⁺ binding [28]. Because Trp residues are dispersed over the whole molecule, the results favor the idea that binding of Na⁺, and probably other ligands to thrombin is accompanied by global effects on thrombin structure [28], which may not be detected from the crystal structure or mutagenesis. Although the changes in flexibility have been demonstrated clearly, which structural differences are involved in thrombin function and the mechanisms are at present unclear.

Exosites in the specificity of other blood coagulation proteinases

Factors Xa, IXa, VIIa, and activated protein C (APC) have topologically similar exosites I or II in their catalytic domains, which differ significantly in sequence [8]. Exosite I in these

proteinases is the site of Ca^{2+} binding, which is absent from thrombin due to insertion of the K70 side chain into this site. Consequently, these proteinases are regulated by Ca^{2+} , which is linked to Na^+ binding sites in similar locations as in thrombin [65–68]. Exosite II of FXa is important for ProT substrate recognition and for the critical interaction with FVa [69,70] (Fig. 4). Experimental results from a number of studies of FXa–FVa interactions are nicely summarized in the context of a molecular model of the complex [71]. In FIXa and Xa, exosite II also overlaps the heparin binding site, which mediates enhancement of the rate of their inactivation by the antithrombin•heparin complex [72,73]. Exosite II of FIXa mediates its interaction with FVIIIa, which is linked to Ca^{2+} binding [65,74] (Fig. 4). APC, on the other hand, binds heparin through exosite I. The cationic exosite I of APC directs the interaction of APC with FVa, promoting initial cleavage at R506, and accommodating heparin binding that facilitates its inactivation by protein C inhibitor [75–79] (Fig. 4). Unlike thrombin, FXa, IXa, VIIa, and APC contain γ -carboxyglutamic acid domains required for calcium-dependent phospholipid membrane binding, which serves to localize their reactions to physiologic membranes. They also contain two epidermal growth factor-homologous domains, which mediate protein–protein interactions and participate in substrate recognition. Molecular modeling studies of the FXa•FVa, FIXa•VIIIa, and APC•FVa complexes summarize the experimental evidence for these interactions [71,75,80,81]. The auxiliary domains contribute greatly to substrate specificity by controlling the assembly of membrane-bound productive enzyme–cofactor–substrate complexes, which enhance the rates of product formation 1000- to 300,000-fold to the rates required for normal hemostasis [82–84]. Membrane assembly of coagulation complexes involves specific protein–protein interactions that provide the optimum orientation of the complex on the membrane surface and conformational changes due to exosite-mediated interactions critical to regulation of substrate specificity.

Proexosite activation

Exosite I and the Na^+ binding site on ProT are in low affinity, proexosite states that are expressed with the conformational changes that accompany catalytic site activation induced by insertion of the I16 N-terminus into the N-terminal binding pocket and formation of the critical salt bridge with D194 [46,85–89]. There is evidence that proexosite expression also plays a role in FXa, IXa and VIIa interactions. Activation of FX is apparently required for interaction of its exosite II with FVa and for expression of the Na^+ site. These conclusions derive partly from Y225P FXa, which does not bind Na^+ , and exhibits weakened N-terminal insertion that allows it to assume a zymogen-like conformation [90]. The mutant has lower affinity for FVa, suggesting that FX binds weakly to FVa, and cofactor affinity is positively linked to Na^+ binding [90]. Molecular dynamics studies of a FX model and FXa structure found major differences between the zymogen and proteinase catalytic domains in the Na^+ and Ca^{2+} binding sites and a segment that participates in FVa binding, indicating that these sites are expressed by FX activation [91]. Direct studies of FX and FXa binding to FVa in solution and on platelets support proexosite expression in the observation that FX activation results in a >60-fold increase in affinity [92,93]. Based on structural studies of prethrombin 2 (Pre 2) [94] and FVII and VIIa [68,95] indicating the lack of formation of the Na^+ binding site on both zymogens, and the Ca^{2+} binding site in the FVII proteinase domain [96], it was suggested that activation of other coagulation factors may be accompanied by formation of functional Na^+ binding sites [90]. This suggests further that exosites on these enzymes linked to Na^+ binding may also be present in proexosite states on the zymogens, with expression accompanying formation of the active proteinase.

The case of FIX appears similar to that of FX in that the heparin binding site (exosite II) overlaps segments mediating FVIIIa binding [73,97,98] (Fig. 4). Molecular modeling studies of the FIXa•VIIIa complex summarize the experimental evidence for these interactions [80]. Factor IXa binds tightly to platelets in the presence of FX and FVIIIa, but this is not diminished by a

large excess of FIX, suggesting that the FVIIIa binding site is expressed on activation of FIX [99]. This is supported further by the demonstration that FIX activation is associated with a *c.* 28-fold increase in affinity for the FVIIIa light chain [100]. Similar to FX, disabling of the Na⁺ site by Y225P substitution weakens N-terminal insertion, affinity for FVIIIa, and catalytic efficiency, indicating that these functions are linked to the zymogen-proteinase transition [65]. There is scant evidence concerning whether exosite I of APC is in a proexosite state in the zymogen. Na⁺ binding to protein C could not be detected in intrinsic fluorescence studies [101], suggesting that this site is not formed. Using an inserted Trp residue as a reporter, however, Rezaie and co-workers [102] found that Ca²⁺ and Na⁺ binding were linked in the zymogen, with affinities for Na⁺ reduced 5- to 8-fold and Ca²⁺ increased 4-fold compared with APC [74,102]. Factor VIIa is an incompletely activated proteinase due to weak N-terminal insertion, which is induced by the cofactor, tissue factor (TF), through extended exosite interactions, including the FVIIa proteinase domain [96,103]. Mutations of certain residues in the D194 pocket of FVIIa that stabilize insertion increase TF affinity for FVIIa but not the zymogen, suggesting that the activating conformational change is required for allosteric regulation of TF affinity [104]. Factor VIIa has an electronegative cluster of residues in the catalytic domain in a position similar to the electropositive exosite I of thrombin, which play a key role in recognition of FX as a substrate [103,105,106] (Fig. 4). It has been proposed that TF stabilization of the inserted N-terminus is linked to restructuring of surface exosite residues for optimum substrate interactions [103]. A monoclonal antibody raised against the FVII zymogen and directed at the substrate-binding exosite binds with lower affinity to FVIIa compared with the zymogen, possibly reflecting expression of the exosite [103,106]. The kinetic pathway of blood coagulation zymogen activation is governed by exosite-driven assembly of coagulation complexes. Assembly of the FXa•Va and FIXa•VIIIa catalytic complexes are doubly regulated by the requirement for the activated protein cofactor as well as the active proteinase. The requirement for exosite expression coupled to catalytic activity ensures that the proteinase, activated cofactor and substrate are in the optimum conformations to catalyze the activation reaction specifically, and restricts premature activation of blood coagulation.

Thrombin exosite expression during ProT activation

ProT activation is accompanied by expression of exosites I and II on thrombin, as well as linkage between Na⁺ binding and exosite I, and the catalytic site. ProT is activated by sequential cleavage of two sites by the FXa•Va•membrane (prothrombinase) complex (described in an excellent review by Krishnaswamy [107]) (Fig. 5). Initial cleavage occurs at R320 in the catalytic domain, activating the catalytic site in the formation of meizothrombin (MzT). This is followed by cleavage at R271 between the catalytic and F2 domains, generating the free thrombin and fragment 1.2 (F1.2) activation products. In the absence of FVa, cleavage occurs in the alternative order, with slow R271 cleavage resulting in the inactive Pre 2•F1.2 non-covalent complex, followed by cleavage of R320 to form active thrombin. When thrombin is generated *in vitro* in the absence of thrombin inhibitors it cleaves ProT at R155, releasing fragment 1 (F1) and generating the zymogen form, prothrombin 1 (Pre 1). Pre 1 does not bind to membranes but is cleaved by prothrombinase to the corresponding alternative activation intermediates, meizothrombin-des-fragment 1 (MzT(-F1)) and the Pre 2•F2 complex, and subsequently to thrombin and F2.

Evidence for the presence of proexosite I on ProT was obtained initially from studies of a fluorescein-labeled exosite I-specific hirudin-(53–64) peptide, demonstrating low affinity for ProT and expression of higher affinity on the activation intermediates and thrombin [87]. NMR studies indicated a very low, but detectable affinity (K_D *c.* 500 μ M) of ProT for *N*-acetyl-hirudin-(55–65) [108]. An antiexosite I monoclonal antibody and TM also demonstrated no detectable binding to ProT compared with thrombin, which was not changed on the reaction

intermediates [88]. The pathway of exosite I expression was defined in equilibrium binding studies employing a fluorescein-labeled derivative of Y63-sulfated hirudin-(54–65) to quantitate the status of (pro)exosite I on ProT, Pre 1, and their activation intermediates [46, 85,86,109]. The results of these studies are summarized in Fig. 6. The affinity of ProT for the hirudin peptide (K_D *c.* 3 μ M) is *c.* hundredfold lower than that of free thrombin, demonstrating overall expression of the exosite. The exosite activation pathway occurs with an increase in affinity accompanied by cleavage at R320 and activation of the catalytic site in MzT, whereas the zymogen intermediate, Pre 2•F1.2 binds with low affinity (K_D *c.* 1.3 μ M). On this basis, the conformational changes accompanying the zymogen to proteinase transition result in expression of exosite I. Parallel studies comparing ProT with Pre 1 and its activation intermediates demonstrated an unexpected effect of the F1 domain in modulating proexosite I affinity (Fig. 6) [85]. Pre 1 binds the hirudin peptide with *c.* 7-fold higher affinity than does ProT, suggesting an interaction between F1 and the catalytic domain that attenuates affinity for the peptide [85,86]. Pre 1 and Pre 2•F2 have the same affinity for the peptide, which is enhanced *c.* twentyfold on formation of MzT(-F1) and thrombin [86]. These two effects account for the overall *c.* hundredfold increase in exosite I affinity accompanying ProT activation.

Expression of exosite II was characterized from the binding of F2 and F1.2 to Pre 2 and thrombin [84,85]. Exosite II is covered by the F2 domain and is inaccessible on ProT, Pre 1, MzT, and MzT(-F1). Unlike exosite I, the relatively low affinity of exosite II on Pre 2 for F2 (K_D *c.* 5 μ M) is not affected significantly by the zymogen to proteinase transition, indicating its exposure accompanying dissociation of F2 (Fig. 6). Additional evidence for a modulatory role of the F1 domain was the *c.* sixteenfold higher affinity of F1.2 for Pre 2 compared with F2, and an associated *c.* 3-fold lower affinity of exosite I for the hirudin peptide [85]. The effect of F1 is specific for the zymogen as shown by the normalization of the affinities of F1.2 and F2 for thrombin. This is in contrast to exosite I expression on catalytic domain activation, in that the modulating effect of F1 on exosite II-mediated F1.2 binding is lost on activation of the catalytic site.

To determine whether (pro)exosite I on ProT zymogen species is allosterically regulated by Na^+ , as it is for thrombin, the effect of Na^+ on (pro)exosite I affinity for the fluorescein-labeled hirudin peptide was quantitated for ProT and Pre 1 and their activation products (*J Biol Chem*, in press). Thermodynamic linkage between Na^+ and (pro)exosite I affinity is undetectable for the zymogen forms, ProT, Pre 1, and Pre 2, and is expressed in concert with formation of the catalytic site on MzT, MzT(-F1), and thrombin (Fig. 6). The disrupted structure of the Na^+ binding site in the crystal structure of the Pre 2–hirudin peptide complex demonstrates that the lack of linkage in the zymogens is due to the Na^+ site not being formed, while it is activated as part of the conformational change in the zymogen to proteinase transition [94,110]. The implications of the finding of Na^+ -exosite I hirudin peptide linkage and enhancements of catalytic activity for all of the active species is that the substrate specificity of MzT for membrane-dependent FV and FVIII activation [57,111] and accelerated protein C activation when bound to TM [112,113] are also likely to be Na^+ regulated, perhaps physiologically. The studies of exosite and regulatory site expression for ProT species demonstrate that all such sites are expressed as part of the zymogen to proteinase transition, tightly coupled to the generation of catalytic activity. An exception is that the intramolecular interaction of the F1 domain that attenuates proexosite I affinity is lost on formation of the catalytic site.

Exosites in recognition of substrates by coagulation proteinase–cofactor complexes

The formation of membrane-bound catalytic complexes among coagulation proteinases, their protein cofactors, and substrates is a central mechanism controlling blood coagulation substrate specificity [82–84]. Membrane complex assembly enhances specific substrate recognition by promoting the protein–protein interactions. Rapid-reaction kinetic studies of prothrombinase assembly on phospholipid vesicles showed that FXa and FVa bind rapidly and independently to the membrane, followed by formation of the complex by diffusion on the surface [114]. In the context of the electrostatic searching mechanism of protein–protein interactions, binding of the proteinase, cofactor and substrate orthogonally to the membrane surface reduces greatly the number of non-specific interactions required to form the FXa•FVa•ProT productive complex.

The most intensively studied exosite-driven mechanism of substrate recognition by coagulation complexes is that of prothrombinase-catalyzed thrombin formation. Krishnaswamy *et al.* have amassed compelling thermodynamic, kinetic and structural evidence for a two-step substrate recognition mechanism [107,115–122]. ProT initially docks to exosites on the FXa–FVa-membrane complex, forming a complex in which the active site of FXa remains accessible to small molecule substrates and inhibitors. This is followed by a conformational change in the ternary complex that engages the catalytic site of FXa for cleavage. The significance of this mechanism is that the apparent k_{cat} and K_{m} for the individual cleavages of ProT activation can depend on both the exosite binding and conformational change steps [107]. This mimics the well-established fact that FVa affects primarily k_{cat} and not K_{m} for thrombin formation. Analysis of the kinetics of the four individual ProT cleavages reveals that all substrates, ProT, Pre 2•F1.2, and MzT, have similar K_{m} values and compete for the same exosite on prothrombinase [107,117,120,122] (see Fig. 5). Three of the four reactions also have indistinguishable k_{cat} values, whereas cleavage of ProT at R271 proceeds with a *c.* thirtyfold lower k_{cat} . This is a consequence of the locking conformational change being highly unfavorable for cleavage at R320 in Pre 2•F1.2, whereas the conformational equilibrium for MzT cleavage at R271 is favorable [107].

While there is ample evidence that exosites expressed on FXa contribute substantially to substrate recognition, the role of FVa in the mechanism is to induce their expression, without direct participation of the well-established ProT interactions with FVa in substrate recognition. ProT and its activation products bind specifically to the heavy chain subunit of FVa [47,56, 123,124], but the role of this interaction in substrate recognition is not yet clear. Thrombin and MzT(-F1) bind to FVa in interactions that are exosite I-dependent based on their competitive displacement with the hirudin peptide [47,56] (and unpublished results). This originated the proposed role for ProT–FVa interactions in substrate specificity through (pro)exosite I interactions with FVa in the assembled complex. In the absence of membranes, binding of the hirudin peptide specifically and completely inhibits FVa-acceleration of ProT and Pre 1 activation through a mechanism in which the peptide binds to the free substrates in competition with their productive interactions with the FXa•FVa complex [109]. The simplest explanation in the context of the two-step model is that proexosite I on ProT either mediates directly initial binding to sites on FVa or modulates the conformational equilibrium engaging the FXa catalytic site. In agreement with this hypothesis, mutations in proexosite I residues of Pre 1 impair prothrombinase-catalyzed Pre 1 activation in parallel with loss of inhibition by hirudin peptide [125]. Additional residues on ProT contributing to FVa interactions were identified adjacent to proexosite I [126]. The two sulfated hirudin peptide-homologous sequences between residues 680 and 709 at the C-terminus of the FVa heavy chain were postulated to be the site of proexosite I interaction based on its specificity [46,47,109,127,128]. This is supported by studies of recombinant FVa with substitutions in the heavy chain and inhibition of FVa-accelerated ProT activation by other exosite I-specific ligands [127,129]. This

interpretation is somewhat controversial, however, because an independent investigation of FVa mutants showed no effect of deletion of the hirudin-like sequences on the kinetics of prothrombinase-catalyzed ProT activation [130]. The initial studies of inhibition of FVa activity by the hirudin peptide were done in the absence of phospholipid membranes, which counteracted inhibition by the peptide [109]. Our subsequent unpublished fluorescence studies of FVa binding to active site-fluorescently labeled FXa on lipid vesicles characterized the binding of native ProT to the labeled prothrombinase complex to form the inactive enzyme-cofactor-substrate complex. ProT binds to the assembled FXa•Va complex and its binding is competitively inhibited by the hirudin peptide. These unpublished results provide evidence that (pro)exosite I plays an important role in substrate recognition by the membrane-bound prothrombinase complex. This mechanism is compatible with the substrate recognition mechanism of Krishnaswamy involving exosites on FXa, with the addition of ProT species interacting with FVa in proexosite I-dependent events that also contribute to substrate recognition and catalysis.

As pointed out by Krishnaswamy [107,118], the role of proexosite I in binding of all substrates and products to a common exosite on prothrombinase is apparently incompatible with one previous observation. Thrombin acts as an exosite-directed competitive inhibitor of FVa-accelerated Pre 2 activation. The hirudin peptide inhibits greatly the activation of Pre 2 and blocks competitive inhibition by thrombin, supporting the proexosite mechanism [118]. However, studies with chymotryptic fragments, ζ -1 and ζ -2, containing exosite I and most of exosite II [131], respectively, demonstrated that ζ -2, lacking exosite I, inhibited Pre 2 activation with the same affinity as thrombin, and the ζ -1 fragment did not inhibit Pre 2 activation [118]. These results argue that proexosite I is not required for Pre 2 activation or product inhibition of FVa-accelerated Pre 2 activation. This discrepancy between thrombin product inhibition and hirudin peptide inhibition was suggested to reflect indirect conformational changes in Pre 2 or thrombin by peptide binding that participate in substrate-prothrombinase interactions, rather than competition for exosites that directly mediate binding [118]. Although inhibition by exosite I ligands is clearly established in a number of studies, the role of FVa-ProT interactions and (pro)exosite I in mediating exosite docking and/or affecting the conformational locking step of the substrate recognition mechanism remains to be clarified.

The two-step docking and locking mechanism has been shown to be fairly general, accounting for the kinetics of FX activation by the FVIIa•TF complex [132] and activation of FIX by FXIa [133]. An exception is protein C activation by the thrombin•TM complex, which exhibits a major role for active site interactions in substrate recognition [134].

Exosite switching in regulation of the ProT activation pathway

Recent studies of the source of the ordered cleavages of ProT by prothrombinase have revealed the predominant role of substrate interactions in directing the pathway [107,115]. The pathway through cleavage at R320 to MzT is favored at the expense of Pre 2•F1.2 formation by a thirtyfold lower k_{cat} for the latter reaction [122]. Cleavage of the identical site in MzT to form thrombin proceeds with a thirtyfold higher k_{cat} . Studies of the activation pathway support a mechanism in which substrates in the zymogen and proteinase states bind in alternate conformations that direct sequential cleavage at the two activation sites by FXa [107,115]. The ProT zymogen is positioned for optimal cleavage at R320, whereas the product, MzT in the proteinase conformation ratchets to a different bound conformation for cleavage at R271. Transition of MzT into the active proteinase state is required for optimal cleavage at R271, as shown in studies of a ProT mutant in which the N-terminal sequence generated by cleavage at R320 was replaced by TAT, which prevents the N-terminal insertion required for activity [115]. The mutant ProT was cleaved normally at R320, whereas its subsequent conversion to thrombin was decreased *c.* twentyfold, and the rate of cleavage at R271 was rescued by

stabilization of the active form with a reversible active site inhibitor. Covalent labeling of the catalytic site of a ProT mutant that could only be cleaved at R271 increased the rate of cleavage *c.* twelvefold [115]. This is thought to be due to occupation of the catalytic site by the covalent transition state analog changing the conformation of the zymogen catalytic domain toward a more active-like conformation. In agreement with this mechanism, active site labeling of ProT alters the ProT activation pathway, inhibiting accumulation of MzT and enhancing formation of the inactive Pre 2•F1.2 intermediate (unpublished results).

The activating conformational change to form MzT is accompanied by expression of increased exosite I affinity and Na⁺-exosite I linkage. The evidence supporting a role for (pro)exosite I in FVa interactions as part of substrate recognition suggests that the proexosite I to exosite I transition may control the bound substrate conformation, ratcheting it from initial cleavage at R320 in the zymogen to R271 cleavage in the active intermediate. This mechanism is compatible with the current model of substrate recognition and ratcheting, with the additional feature that (pro)exosite I-dependent FVa interactions are postulated to determine the alternate bound substrate conformations.

Exosite-targeted anticoagulants

The emergent predominance of exosite-mediated interactions in driving coagulation proteinase substrate specificity has led to several strategies for exosite-targeted anticoagulants. Inhibitors of ProT or thrombin (pro)exosite I, such as the hirudin peptide, an exosite I-specific DNA aptamer, and DYDYQ have been speculated to be potentially useful [57,127,129,135]. Based on the properties of exosites, and their multifunctionality for binding of various structurally different ligands via spatially overlapping residues, it has not yet been possible to identify a ligand that inhibits one but not all interactions of each exosite. Toward this goal, new computational approaches are being developed to identify hot spots and potential binding clefts, and docking approaches that address explicitly the flexibility and water-mediated interactions inherent in protein-protein interactions [136].

Exosite inhibitors derived from snake venoms and hematophagous organisms have been identified as possible anticoagulants [7,19,137]. Bothrojaracin does not block the catalytic site but interacts with both thrombin exosites and with high affinity to proexosite I on ProT, resulting in inhibition of thrombin generation [138,139]. NAPc2 is a flexible protein from the nematode *Ancylostoma caninum* that binds very specifically to FXa as an exosite-directed inhibitor of ProT activation, which is unaffected by incorporation of FXa into the prothrombinase complex [140,141]. The NAPc2•FXa complex is a potent inhibitor of the FVIIa•TF complex [142], which is the basis for its assessment in clinical trials as a promising new exosite-directed anticoagulant.

As a consequence of the different sets of residues involved in overlapping exosite interactions, site-directed mutation of one or two exosite residues can knock out particular interactions while preserving others. Substitution K60fE reduces fibrinogen clotting activity *c.* 6-fold while accelerating protein C activation in the presence of TM *c.* 2.5-fold [31]. R75E substitution of thrombin on the other hand has the converse effects, preserving activity with fibrinogen and not protein C. W215A substitution selectively inhibits Na⁺ binding and fibrinogen clotting activity, while having smaller effects on protein C activation and PAR-1 cleavage [143]. Similarly, thrombin E217A and E217K have no fibrinogen clotting activity but retain 50% activity in protein C activation [144,145], and E217A thrombin has anticoagulant activity *in vivo* [144]. The structure of thrombin E217K shows that the source of its low activity is disruption of the Na⁺ site and resultant collapse into an inhibited conformation, which is apparently restored by TM binding [23]. As a result of these findings, a two-residue substitution mutant (W215A/E217A) of thrombin was developed that has thirty-five thousandfold reduced

catalytic activity toward certain chromogenic substrates, twenty thousandfold reduced activity with fibrinogen, and thousandfold reduced activity toward PAR-1, but develops normal activity in activation of protein C induced by binding to TM [146,147]. The structure of this mutant also demonstrated a disordered Na⁺ site and occluded S1 site in addition to an inactive arrangement of catalytic site residues [147,148]. This mutant has substantial anticoagulant and antithrombotic activity *in vivo* [149]. In a related approach, the murine ProT mutant R157A/R268A, which can only be cleaved by prothrombinase to a stable form of MzT, was developed based on the intrinsically low activity of MzT toward fibrinogen and platelet activation in contrast to its potency as a protein C activator when bound to TM [150]. This mutant has significant anticoagulant activity in a mouse model of thrombosis. Such ProT and thrombin mutants with selective functional inactivation show considerable promise as sources of novel exosite-based anticoagulants.

Active site-blocked coagulation proteinases have been proposed as exosite-directed anticoagulants. Active site-blocked FXa and FIXa, and an inactive TF mutant, are antithrombotic *in vivo*, with minimal effects on normal hemostasis compared with heparin [151]. In this vein, ProT labeled at the catalytic site with FPR-CH₂Cl has anticoagulant and antithrombotic activity (unpublished results). In a mouse model of thrombosis, FPR-ProT decreases the rate of thrombus growth and size. FPR-ProT and S195A ProT have similar properties as competitive inhibitors of thrombin generation in a system of purified proteins. In platelet-rich and platelet-poor plasma, however, FPR-ProT increases the lag time in FVIIa-TF-initiated thrombin generation and reduces the maximum level of thrombin formed, whereas S195A ProT has a much smaller effect. As a result of active site labeling, the pathway of FPR-ProT cleavage by prothrombinase is shifted toward generation of the inactive Pre 2•F1.2 intermediate and away from the MzT pathway, whereas S195A ProT is cleaved normally. The mechanism of inhibition is thought to reflect FPR-ProT acting as a competing alternate substrate of prothrombinase. This illustrates an anticoagulant mechanism based on perturbation of the exosite-directed pathway of ProT activation.

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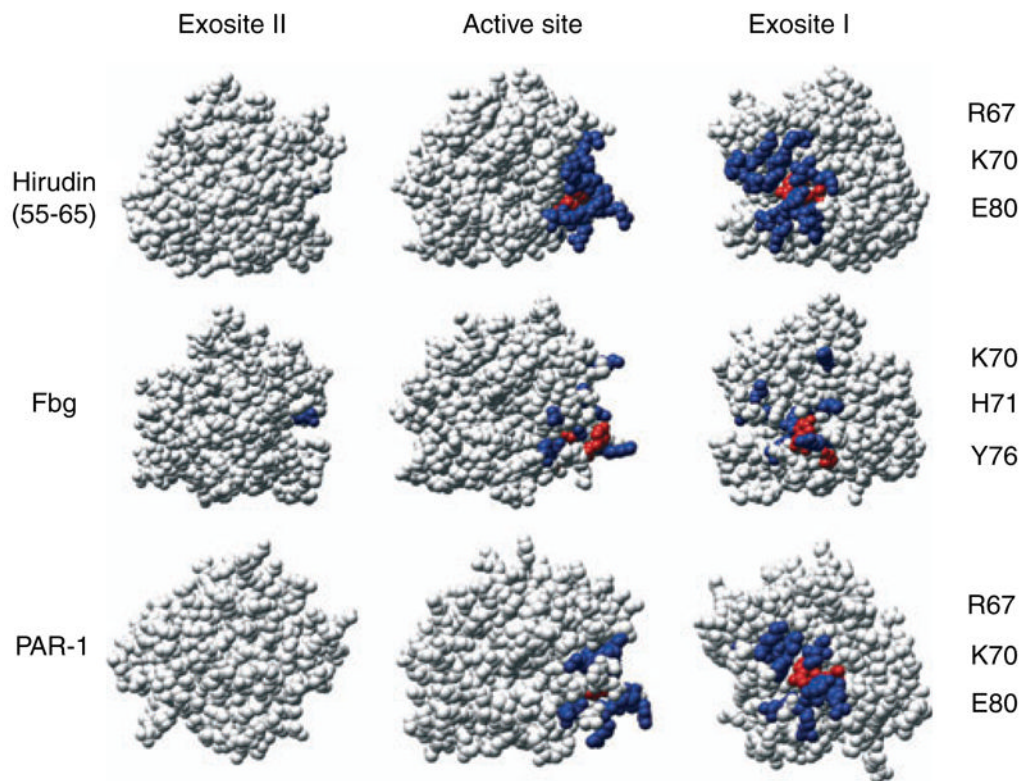
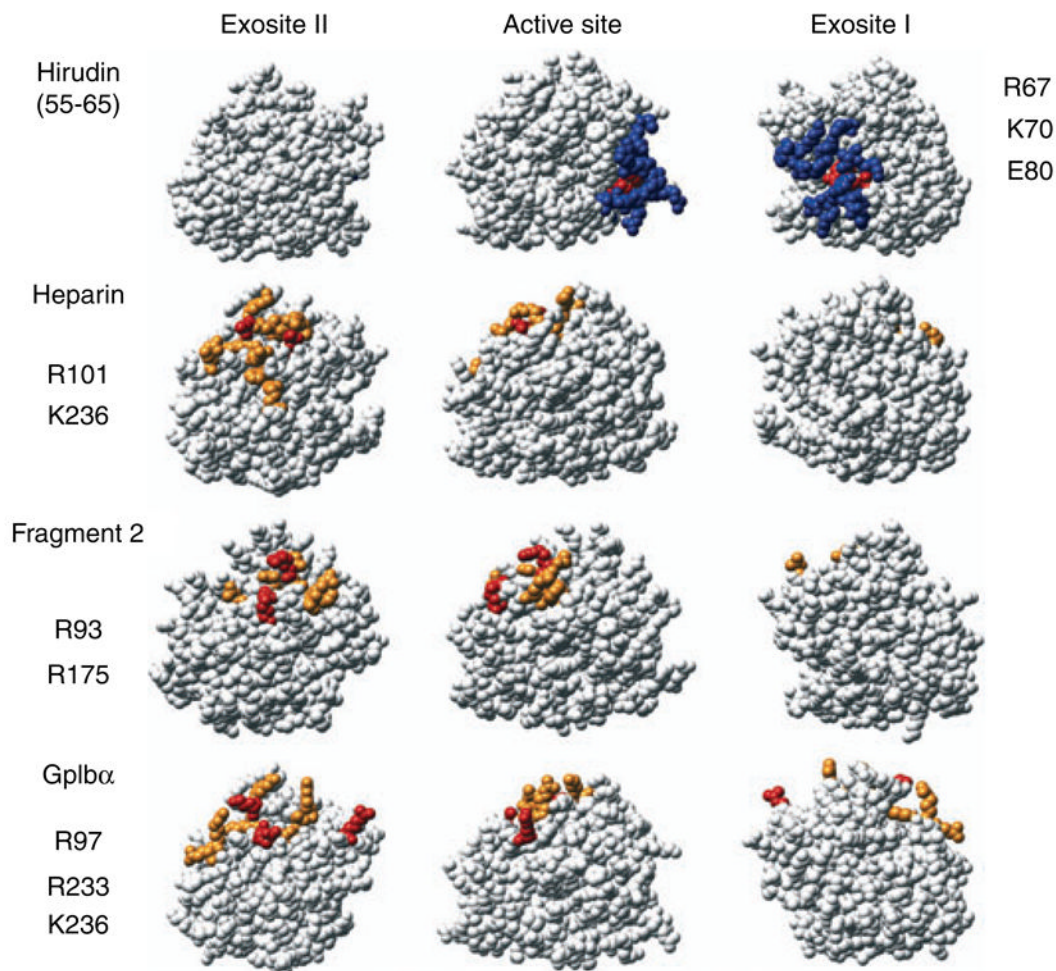


Fig. 1. Functional mapping of thrombin exosite I residues involved in recognition of protein substrates. Thrombin structures are shown in standard Bode orientation [13] (middle) with the active site in the center, facing the reader. Two additional views are shown: *c.* 90° counter-clockwise rotation for exosite I (right), and *c.* 90° clockwise rotation for exosite II (left). Residues that cause at least a 2.3-fold effect by alanine-scanning mutagenesis are shown for exosite I (blue). For Y63-sulfated hirudin-(55–65): F34, K36, S36a, P37, Q38, L65, R67, K70, H71, R73, T74, R75, R77a, E80, E81, I82, M84, Y76, and E77a. For fibrinogen fragment E (*Fbg*): K36, R67, R73, K70, H71, Y76, R73, R77a, K81, and K110. For PAR-1: F34, R67, K70, R73, R75, Y76, R77a, E80, and I82. Residues that displayed the largest fold effect of alanine substitution are shown in red and listed on the right. The structures shown are those determined from thrombin complexes with Y63-sulfated hirudin peptide-(55–65) (1AFE), fibrinogen fragment E (1QVH), and PAR-1 (1NRN).

**Fig. 2.**

Functional mapping of thrombin exosite II residues involved in cofactor binding. Thrombin structures as described in Fig. 1, with the residues for interaction of Y63-sulfated hirudin-(55–65) shown as a reference. Residues identified by alanine-scanning mutagenesis as described in Fig. 1 for exosite I (blue) and exosite II (orange), with the exception of F2, where residues shown are those with the largest number of contacts in the crystal structure. For heparin: H91, R93, R101, R126, R165, H230, R233, K236, W237, and K240. For F2: P92, R93, Y94, W96, R97, R101, R175, and D178. For GpIb α , mutagenesis data indicated: R93, R97, R 101, R233, K236, and K240 [43], while the Dumas structure [40] also indicated direct contacts with R126, E127, N179, and K235. Red residues indicate the largest effect of alanine substitution or the largest number of contacts for F2, and are listed for exosite I (right) and exosite II (left). The structures of thrombin shown were those determined for the complexes with Y63-sulfated hirudin-(55–65) (1AFE), heparin (1XMN), F2 (2HPQ), and GpIb α (1P8V).

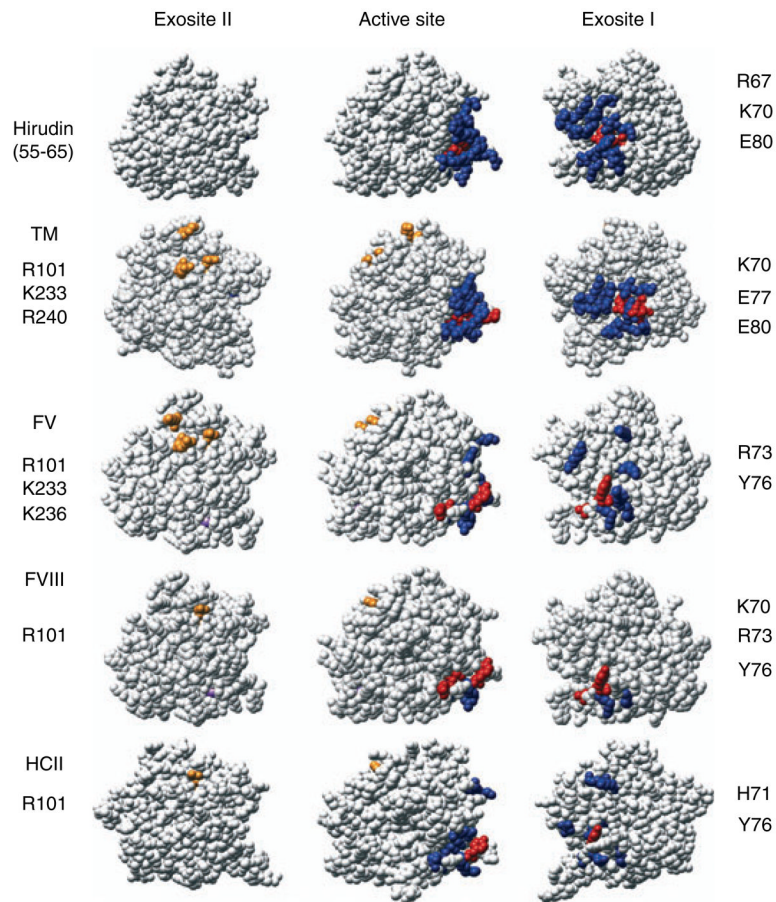


Fig. 3. Functional mapping of thrombin ligands that use both exosites. Thrombin structures are shown in the same orientations as in Fig. 1, with residues involved in the complex with Y63-sulfated hirudin-(55–65) shown as a reference. Residues identified by alanine substitution for exosite I (blue) and exosite II (orange). For TM exosite I: F34, K36, P37, Q38a, L65, R67, K70, H71, R73, T74, R75, Y76, E77, R77a, E80, K81, I82, and K109, and in exosite II residues that interact with the chondroitin sulfate moiety: R101, R233, and K240. For factor V (FV) exosite I: K36, H71, R73, R75, Y76, R77a, K81, and exosite II: R101, R233, and K236. For factor VIII (FVIII) exosite I: K36, K70, H71, R73, R75, Y76 and exosite II: R101. For heparin cofactor II (HCII) exosite I: Q38, R67, K70, H71, R73, R75, Y76, K109, and K110, and in exosite II R101 is involved in heparin acceleration. Red residues are those displaying the largest effects of alanine substitution, which are listed for exosite I (right) and exosite II (left). The structures of thrombin shown were those determined for Y63-sulfated hirudin-(55–65) (1AFE), TM epidermal growth factor domain fragment-3,4,5 (1DX5), and heparin cofactor II (HCII) (1JMO). For FV and FVIII, where structures are unavailable, the mutagenesis data are shown on the fast form of thrombin with Na⁺ in purple (1SFQ).

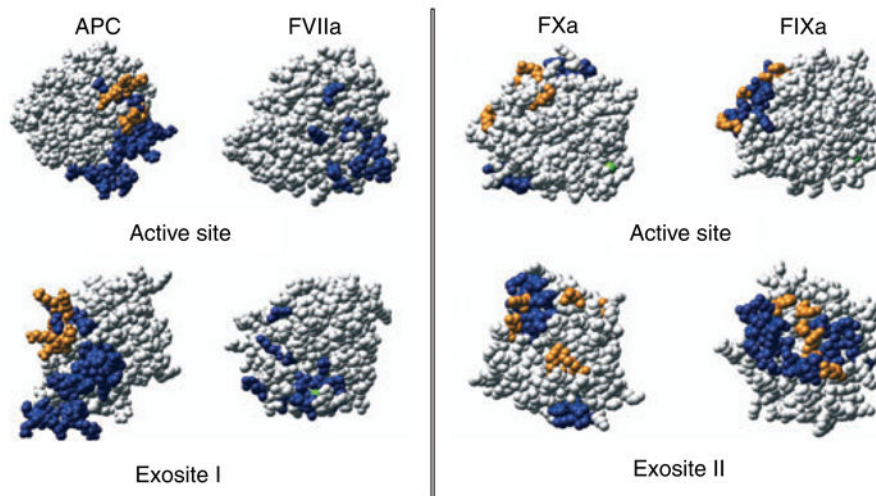


Fig. 4.

Comparison of exosite I and II locations on homologous proteinases, thrombin, factor Xa, APC, factor IXa, and factor VIIa. Structures are shown in standard Bode orientation (above) or turned to the relevant exosite, as indicated (below). Residues are colored blue for cofactor or substrate interactions and orange for the heparin binding site. For factor Xa (FXa): orange residues are R93, K96, R125, R165, K169, K236, and R240 [72]. Factor Xa residues involved in factor Va binding include all orange residues and blue residues D185-D189 [152] and V231-R245 [153]. For APC: orange heparin binding residues are K37, K38, K39, E60a, S61, K62, and K63 [77]. Blue residues involved in factor Va substrate recognition [154,155] are, S36-K39 (37 loop), D60-R67 (60 loop), E70-E80 (70–80 loop), and R147-R153 (autolysis loop) [156]. For factor IXa (FIXa): orange residues are R126, R165, R170, K230, and R233 and blue residues involved in factor VIIIa binding are K126-G134 (126-helix), L162-R170 (162-helix), F174-N178 [79,80]. For factor VIIa (FVIIa): blue residues involved in factor X substrate recognition are V21, K24, Q40, K60a, V67, E70, D72, L73, S74, E75, D79, E80, R148, T151, L153, E154, M156, T165, and K192 [105] determined by alanine scanning, excluding the residues that may directly impair substrate cleavage and Na⁺ binding [157]. The structures are the catalytic domains of FXa (1LQG), APC (1AUT), FIXa (1RFN), and FVIIa (1DVA).

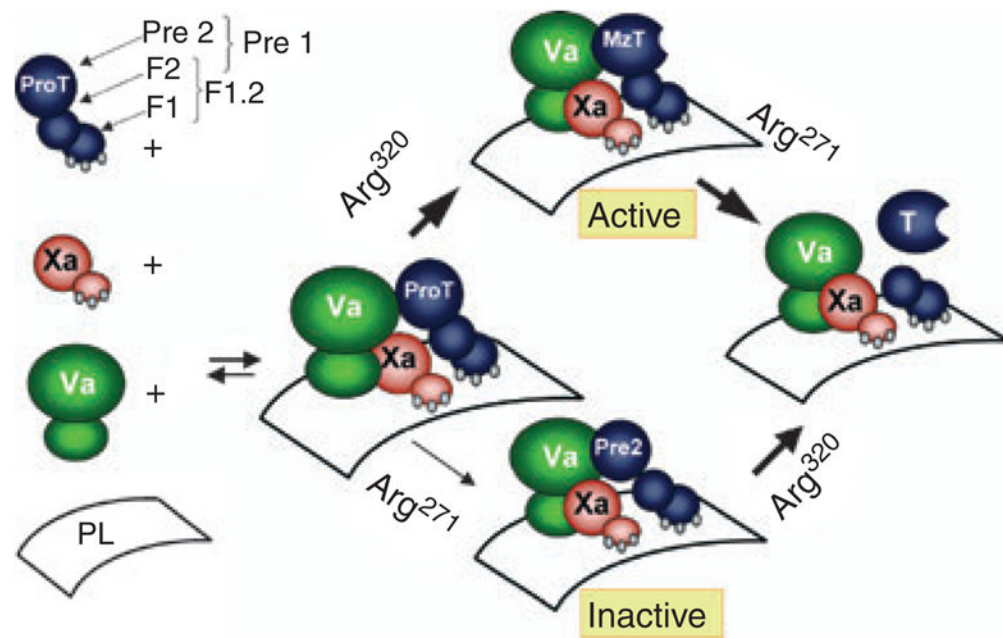


Fig. 5. Mechanism of prothrombin activation. ProT activation is initiated by assembly of ProT, factor Xa (Xa), and factor Va (Va) on a phospholipid membrane surface. In the presence of saturating factor Va, cleavage at R320 in the ProT catalytic domain occurs first, yielding the active MzT reaction intermediate. Subsequent cleavage of MzT at R271 between the catalytic and F2 domains yields the products, thrombin (T) and F1.2. In the absence of factor Va, the alternative cleavage pathway predominates, with slow cleavage of R271 to form the inactive Pre 2•F1.2 non-covalent complex, followed by cleavage at R320 to form the products.

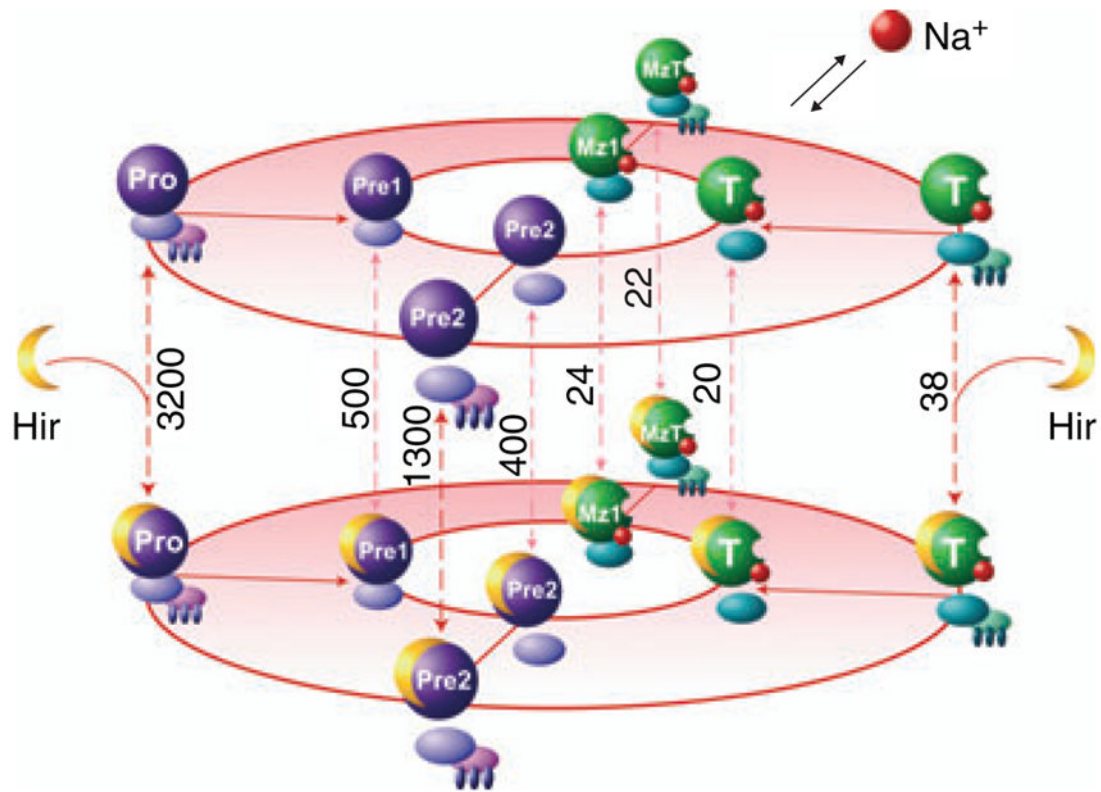


Fig. 6.

Pathways of exosite I and II expression and expression of the Na⁺ binding site during ProT and Pre 1 activation. Pathways of ProT (Pro) activation through the Pre 2•F1.2 or MzT alternative intermediates to the products, T + F1.2 (outer oval) and Pre 1 activation through Pre 2•F2 or MzT(-F1), to T + F2 (inner oval) are shown. Expression of the Na⁺ binding site is illustrated by association of Na⁺ (red spheres) with the intermediates and products. Purple proteins indicate inactive species, while green proteins indicate active forms. Fluorescein-labeled Y63-sulfated hirudin-(54-65) (Hir) binding equilibria are represented vertically (red dashed lines), with the dissociation constants for each species in nM. Modified with permission from Anderson and Bock [85].