Thrombin Formation

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The Setting

Vascular damage exposes tissue factor to flowing blood and triggers a series of proteolytic activation events associated with the tightly regulated hemostatic response. The activation events convert inactive precursors to active proteinases and cofactors that function in concert with cells exposing phosphatidylserine on their outer membrane leaflet and localized at the site of injury [1–6]. Thrombin is the ultimate protease in the clotting cascade (Fig. 4.1) and is responsible for catalyzing blood cell activation and fibrin formation. It also plays a key regulatory role in determining flux through the cascade and its own formation. Low levels of thrombin formed following initiation of the cascade catalyze the proteolytic activation of factors XI, V, and VIII to greatly amplify flux through the cascade. Later in the clotting response, thrombin bound to thrombomodulin on the endothelium catalyzes protein C activation. Activated protein C downregulates flux through the cascade by catalyzing the inactivation of factors Va and VIIIa produced by thrombin early in the clotting response. The clotting process is shut down by the irreversible inhibition of thrombin and the other serine proteinases of coagulation by serine proteinase inhibitors (Serpins), such as antithrombin III, that circulate at high concentrations in plasma and act as suicide substrates. While it is convenient to discuss these details as discrete phases of the coagulation response, the different reactions occur contemporaneously with the net result at any time following vascular damage being determined by the relative contributions of the procoagulant, anticoagulant and inhibition reactions. Resultant clot formation is varyingly determined by the rate, amplitude, and total amount of thrombin produced in its transient production following vascular damage (Fig. 4.2). These features outline, in broad strokes, the key features of the response following vascular damage from a thrombin-centric perspective. The central regulatory role of thrombin in the clotting response emanates from its ability to act with pan-specificity on numerous protein substrates essential for the regulation of coagulation, anticoagulation, fibrinolysis, and cellular responses in blood and the vasculature. Because of its key and opposing roles in the regulation of coagulation, it logically follows that targeting the active site of thrombin for therapeutic

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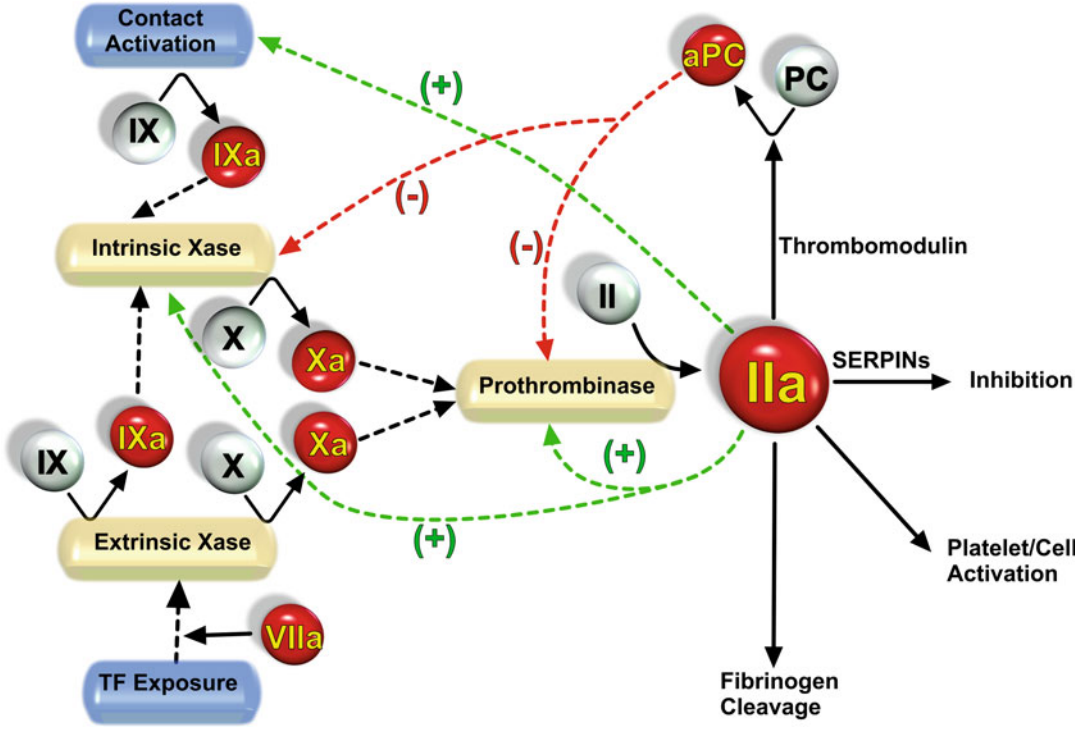

Fig. 4.1 The blood coagulation cascade. The zymogens are colored *grey* and proteinases are colored *red*. Negative feedback reactions are denoted by (–) and *red dashed lines* while positive feedback reactions are denoted by (+) and *green dashes*

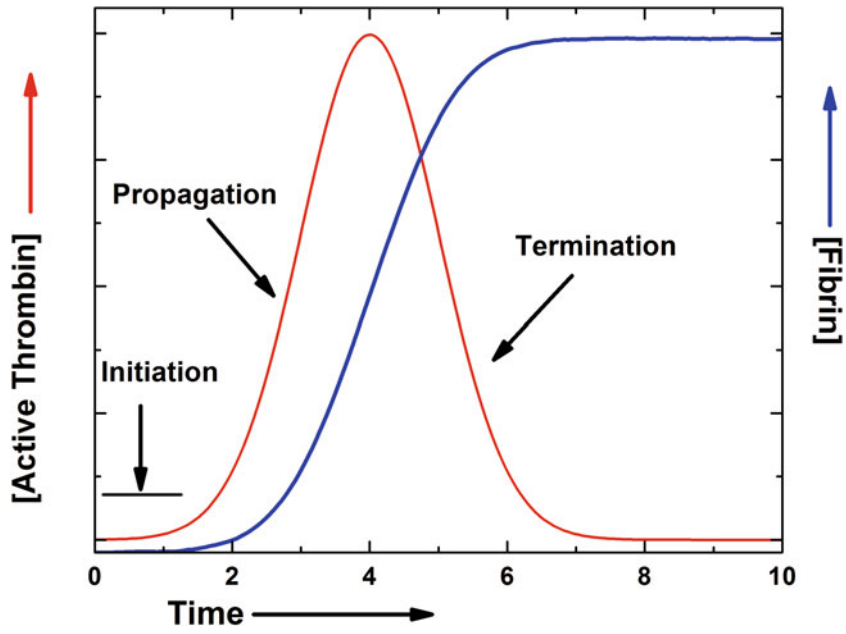

Fig. 4.2 Fate of thrombin following initiation of coagulation. In the initiation phase, positive feedback reactions enhance flux through the cascade to yield an explosive increase in thrombin formation in the propagation phase. Termination results following initiation of the anticoagulant reactions and elimination of proteinases by reaction with serpins. Fibrin is expected to evolve as the first derivative of the thrombin trace provided fibrinogen consumption is minimal

gain with inhibitors will carry significant risk for dysregulation of the hemostatic response. A second logical problem posed by therapeutic targeting of the active site of any of the coagulation serine proteinases relates to the potentially harmful and unpredictable consequences of shielding the proteinase in a reversibly inhibited complex that is protected from irreversible inhibition and removal by Serpins.

The Catalyst for Thrombin Formation

The conversion of prothrombin to thrombin represents the first committed step in coagulation as it is the first nonredundant activation reaction in the pathway between the initiation of coagulation and thrombin production (Fig. 4.1). This imbues the catalyst for thrombin formation with key functional and regulatory significance for coagulation, evident from severe bleeding in patients with defects in the catalyst [7]. Unlike thrombin, which shows relatively broad substrate specificity, the other serine proteinases of coagulation are far more selective for their substrates. Their specificity and selectivity arises from the membrane-dependent association of cofactors with their respective proteinases. This architecture is exemplified by the prothrombinase complex responsible for catalyzing the conversion of prothrombin to thrombin. Prothrombinase is composed of a complex of the serine proteinase factor Xa, and its cofactor, factor Va, that assembles through reversible protein–protein and protein–membrane interactions on membranes containing phosphatidylserine (Fig. 4.3). A wealth of biochemical and biophysical insights into the assembly and function of prothrombinase have been derived in the last 30 years from studies using purified proteins, synthetic phospholipid vesicles containing an optimal ratio of phosphatidylcholine and phosphatidylserine (PCPS). Although Xa is a competent serine proteinase with a fully formed active site, it acts as a very poor activator of prothrombin. Addition of saturating concentrations of membranes and Va dramatically improves the steady-state kinetic constants for prothrombin activation (Table 4.1). These improvements in substrate affinity and catalytic power attributed to the effects of membranes and the cofactor result in the proverbial 500,000-fold increase in the rate of thrombin formation at the physiological concentration of prothrombin. This is a generalizable phenomenon as comparable functional changes accompany the assembly of the other coagulation

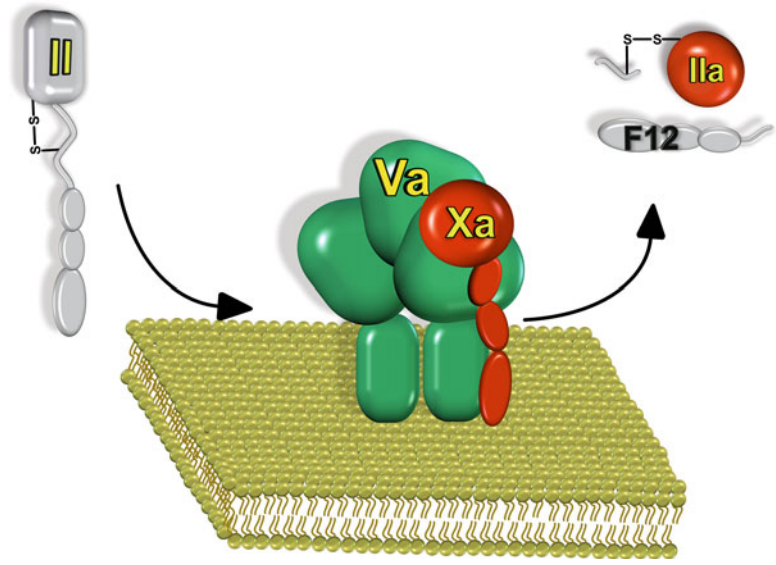

Fig. 4.3 Membrane assembled prothrombinase. Prothrombinase assembles through membrane-dependent interactions between the cofactor (factor Va) and the proteinase (factor Xa) on membranes containing phosphatidylserine. The complex cleaves the zymogen prothrombin (II) to thrombin (IIa) and the activation peptide fragment 1.2 (F12). Taken with permission from reference [65]

Table 4.1 Steady state kinetic constants for prothrombinase

Sustrate		Prothrombin[a]		Peptidyl substrate[b]	
Enzyme species[c]	Km (μM)	Vmax/E_T (s^{-1})	Relative rate[d]	Km (μM)	Vmax/E_T (s^{-1})
Xa	84	0.01	1	98	172
Xa/PCPS	0.7	0.05	203	98	181
Xa/Va/PCPS	0.4	108	512,000	200	179

[a]Kinetic constants for bovine prothrombin cleavage by bovine Xa alone [63], or for the activation of human prothrombin by human enzyme constituents in the presence of membranes [64]
[b]Kinetic constants for the cleavage of Spectrozyme Xa (methoxycarbonyl-D-cyclohexylglycyl-glycyl-L-arginine-p-nitroanilide) taken from reference [35]
[c]The enzyme species correspond to Xa alone, Xa saturably bound to membranes (Xa/PCPS) and Xa assembled into prothrombinase (Xa/Va/PCPS) using saturating concentrations of membranes and Va
[d]Relative rates at 1.4 μM prothrombin calculated from the steady state kinetic constants

enzyme complexes containing homologous constituents that interact in an analogous way [1]. On the one hand, these functional effects are considered essential for highly accelerated zymogen activation limited to the site of vascular damage leading to rapid clot formation. It follows from reason that the paradoxically poor activity of the proteinase alone in activating its biological substrate in the absence of membranes and cofactor is an equally important feature necessary for limiting thrombin formation in the absence or beyond the site at which damaged or activated cells expose phosphatidylserine.

Membranes and Function

All interacting species, proteinase, cofactor and substrate bind reversibly to membranes containing phosphatidylserine. Factor Xa and prothrombin are vitamin K-dependent proteins that contain γ-carboxyglutamic modifications at their N-terminal Gla domains. This domain mediates their binding to membranes in a Ca^{2+}-dependent manner [1]. Unlike the vitamin K dependent proteins, membrane binding by factor Va is not Ca^{2+}-dependent and likely occurs through the two discoidin-like C domains at its C-terminus [1]. Mechanistic details aside, all three interacting species bind to the membrane surface in an oriented fashion which lies at the heart of how membrane binding modulates function.

Factors Xa and Va interact with a physiological irrelevant affinity (Kd ~2 μM) in solution. Enzyme assembly requires the initial binding and independent binding of Xa and Va to the membrane surface followed by their surface-limited interaction to form prothrombinase with ~1000-fold enhanced affinity (Kd ~1 nM) [8, 9]. The efficient interaction on the membrane surface arises from their oriented binding to the membrane and the function of the membrane as a wave guide to limit dimensions and accelerate productive collisions. Linkage between protein–protein and protein–membrane interactions further contributes to enhanced affinity and permits prothrombinase to assemble efficiently at picomolar concentrations, far lower than would be predicted from knowledge of the individual binding constants [9]. These points outline the essential role played by membrane binding by reactants in facilitating prothrombinase assembly.

Because prothrombin can also bind membranes, it follows that similar phenomena must apply to its delivery and cleavage by the membrane assembled enzyme. An influential paper published 30 years ago sought to provide a comprehensive mechanistic interpretation of how membrane binding by prothrombin contributes in a fundamental way to the enhanced function of membrane-bound prothrombinase [10]. In this interpretation, preventing membrane binding by prothrombin was viewed as the functional equivalent of preventing the assembly of prothrombinase leading to catastrophic 3000-fold decrease in rate. It now seems likely to be a considerable over-estimate of the contribution of the substrate–membrane interaction to the rate of thrombin

formation as rate is only decreased by a modest fourfold for a prothrombin variant lacking γ-carboxyglutamic modifications necessary for membrane binding [11]. Conversely, membrane binding plays a major role in the presentation of the substrate to membrane-bound prothrombinase and the regulation of the pathway for substrate cleavage, covered in more detail below.

The perennial criticism of mechanistic inferences drawn from studies using purified proteins and synthetic membranes is the lack of relevance to the situation in vivo wherein the activated platelet adherent at the site of vascular damage has long been considered the most important surface for the assembly and function of prothrombinase [12]. There is little arguing that the platelet surface is complex and likely imposes fundamentally different physical and perhaps chemical constraints on prothrombinase assembly and function than pure phospholipid vesicles containing only phosphatidylcholine and phosphatidylserine. The long-standing suggestion for a specific platelet receptor(s) for the constituents of prothrombinase are yet to be borne out [13]. It now appears increasingly likely that the litany of functional differences ascribed to the platelet can adequately be attributed to the limiting exposure of phosphatidylserine [11]. Recent studies imaging prothrombinase following laser damage to the mouse microcirculation further question the preeminent role ascribed to platelets in supporting enzyme assembly and function. Instead, it appears that the damaged and activated endothelium is the major site supporting the binding of factors Xa and Va [14]. Relating concepts developed from biochemical and biophysical studies in purified systems to explain prothrombinase function on naturally relevant membranes represents a major outstanding challenge in the field.

The Cofactor and Function

The need for a cofactor for efficient thrombin formation has presented a long-standing puzzle in the field. This arises from the fact that the catalytic domain of factor Xa closely resembles trypsin, yet trypsin does not need a cofactor to act on its protein substrates with high efficiency. The early observation that factor Va seemingly increased the rate constant for catalysis for thrombin formation suggested a self-evident mechanism for cofactor function [1]. Factor Va must bind Xa and alter the active site to make it more complementary to the transition state and thereby lowers the free energy barrier for catalysis [15]. While this idea was supported by Va-dependent changes in spectroscopic probes incorporated into the active site of Xa, the unanswered question is whether these perturbations detected at the active site are relevant to function [16, 17]. Studies using a transition state irreversible peptidyl inhibitor and a variety of peptidyl substrates for Xa have failed to provide any evidence to suggest Va-dependent increases in active site function [15]. Indeed, the large increases seen in the kinetics of prothrombin activation upon the assembly of prothrombinase are not accompanied by significant changes in peptidyl substrate cleavage (Table 4.1). This implies that the questions of protein substrate specificity of prothrombinase and the mechanism by which factor Va functions within prothrombinase are closely related questions not properly assessed by peptidyl substrates that target the active site of Xa within prothrombinase. These ideas are further supported by the repeated failure of peptidyl substrate studies to adequately account for the cleavage site preference of prothrombinase (or Xa) within prothrombin [18–20].

Prothrombin Activation

Thrombin formation requires proteolysis at two sites within prothrombin. Consequently, there are two possible pathways for prothrombin activation (Fig. 4.4). Initial cleavage of prothrombin following Arg^{271} yields the N-terminal pro-piece fragment 1.2 (F12) and the zymogen prethrombin 2 (P2), which is then further cleaved following Arg^{320} to yield the disulfide-linked two chain form of thrombin. Cleavage of the bonds in the opposite order yields the proteinase meizothrombin (mIIa) as an intermediate. Its subsequent processing at Arg^{271} yields the final reaction products. The cleavage pathway that predominates is

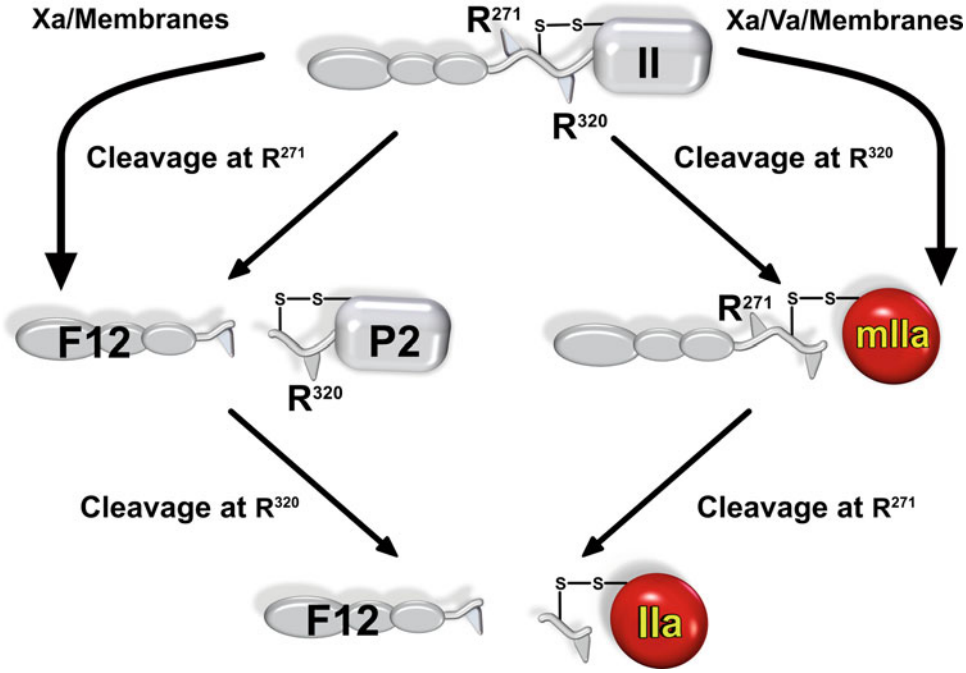

Fig. 4.4 Pathways for prothrombin activation. The conversion of prothrombin to thrombin results from cleavages following Arg^{271} and Arg^{320}. Initial cleavage following Arg^{271} yields the pathway on the *left* and produces the zymogen, prethrombin 2 (P2) and the propiece, fragment 1.2 (F12) as intermediates. P2 requires further processing at Arg^{320} to yield thrombin. The pathway on the *right* arises from initial cleavage following Arg^{320} which produces the proteinase meizothrombin (mIIa) as an intermediate. Further cleavage following Arg^{271} is required to yield IIa and the propiece, F12. The *left arm* of the pathway predominates in the absence of Va while activation almost exclusively proceeds through the *right arm* for prothrombinase. Taken with permission from reference [65]

dependent on the presence of factor Va and either adequate phosphatidylserine content of membranes or the ability of prothrombin to bind the membrane surface [21]. In the absence of factor Va, the reaction proceeds primarily via cleavage at Arg^{271} leading to the formation of P2 and F12 as intermediates. When prothrombinase is assembled with saturating concentrations of Va and membranes with high phosphatidylserine content (25 % w/w), prothrombin activation proceeds essentially exclusively via initial cleavage at Arg^{320} and the formation of meizothrombin as an intermediate [22]. Variable flux towards thrombin formation via the two arms of the pathway is evident when Xa is saturated with very high concentrations of Va in the absence of membranes, when membranes containing 5 % phosphatidylserine are used to assemble prothrombinase, when prothrombinase is assembled on activated platelets versus endothelial cells and when prothrombin with a deficit in γ-carboxyglutamate content is used as a substrate [11].

The biological significance of the pathway of prothrombin activation lies in the fact that P2 is a zymogen while mIIa is a proteinase that is defective in several of the procoagulant activities of thrombin but retains the ability to bind thrombomodulin and function as an anticoagulant by catalyzing protein C activation [23, 24]. The biochemical significance lies in the wealth of insights that this process reveals regarding the substrate specificity of prothrombinase, aspects of the role of cofactor and the substrate–membrane interaction in thrombin formation and the putative importance of the conversion of zymogen to proteinase in regulating the process of prothrombin activation. Because cleavage of precursors at multiple sites, frequently in ordered

fashion, is commonplace in coagulation, prothrombin activation provides a template for the consideration of the complicated enzymology associated with such cleavage reactions.

The interrelationship between substrate specificity and cofactor function is again revealed by the fact that the large increase in catalytic function that occurs with the addition of factor Va is associated with the ability of prothrombinase to distinguish between the two cleavage sites within prothrombin. Thus, while both cleavage sites within prothrombin are available to be cleaved by added proteinase, near-absolute discrimination between the two cleavage sites is evident from the fact that when prothrombin engages prothrombinase only the Arg^{320} site is available for cleavage by the enzyme complex. Cleavage at Arg^{271} only occurs following the formation of mIIa. Because mIIa is a serine proteinase and prothrombin is a zymogen, it follows that the zymogen to proteinase transition plays an important role in determining the sequential action of prothrombinase on prothrombin and its derivatives. Another complexity revealed here is that regardless of the pathway of prothrombin activation, thrombin formation results from the action of prothrombinase or Xa in two sequential enzyme-catalyzed reactions. Thus, steady state kinetic constants measured by adding prothrombin and measuring the rate of thrombin formation cannot be meaningfully interpreted. This suggests fundamental problems in deriving useful mechanistic interpretations using such kinetic measurements that have been used to frame the problem since the early 1980's.

Proteinase Formation

Thrombin and the other serine proteinases of coagulation are members of the S1 peptidase clade of which chymotrypsinogen is the archetypic member [25]. Consequently it is most useful to consider the areas of interest related to proteinase function within the catalytic domain using the homologous numbering system according to chymotrypsinogen (denoted by a c following the residue number, eg. Ile^{16c}) [26]. In the case of prothrombin and its derivatives, cleavage of the Arg^{320}-Ile^{321} peptide bond, which corresponds to Arg^{15c}-Ile^{16c}, is the key step in the conversion of the zymogen to proteinase. Cleavage reveals a new N-terminus in the catalytic domain which inserts in a sequence-specific way into the N-terminal binding cleft and forms a salt-bridge with Asp^{194c}. This process, aptly termed "Molecular Sexuality" by Wolfram Bode, is associated with ordering of four activation domains, 16c-21c, 142c-152c, 184c-194c, and 216c-233c, which are disordered in the zymogen. The net outcome includes correct formation of the primary specificity pocket (S1) which allows the binding of Arg-containing substrates at the active site. There is a flip in the amide bond of Gly^{193c} to form the oxyanion hole which is necessary to stabilize the transition state. These are the primary changes that allow the proteinase product to bind substrates with high affinity and catalyze their cleavage with a greatly increased catalytic rate constant, yielding 10^4–10^5-fold improvements in catalytic function in comparison to the zymogen. Thrombin also utilizes surfaces removed from the catalytic site, termed anion binding exosites (ABE1 and ABE2), to interact with its substrates and ligands (Fig. 4.5). Proteinase formation reorders the 70c loop to mature and enhance the binding function of ABE1. Na^+-binding to a specific site in the proteinase is also enhanced such that the cation is not bound in the zymogen but is part of the stabilized and folded structure of the proteinase. A final observation is that formation of the proteinase destabilizes ligand binding to ABE2 [27].

The most relevant ABE2 ligand for prothrombin activation is the F12 pro-piece that interacts with the proteinase domain through the fragment 2 (F2) region. Because of the destabilizing effect of proteinase formation on ABE2 ligation, the zymogen binds F12 with high affinity (~10^{-8} M), while thrombin binds F12 with ~100-fold weaker affinity [27]. The result of this discrepancy is that F12 greatly enhances the cleavage of P2 at Arg^{320} by binding the zymogen through F2 region and facilitating its binding to membranes through the F1 domain [27]. In contrast, the analogous but weaker interaction between thrombin and

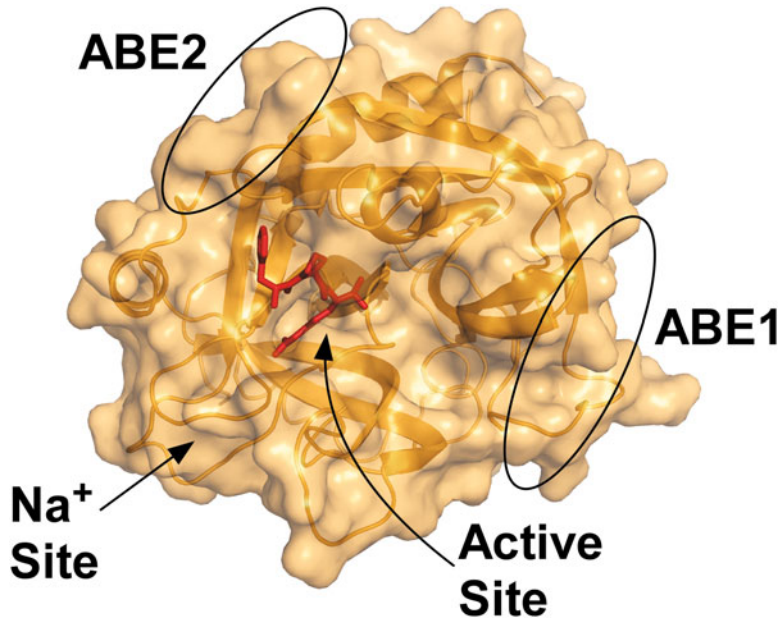

Fig. 4.5 X-ray structure of thrombin. The structure of human thrombin inhibited by D-FPR-chloromethyl ketone (1PPB) is shown in the standard orientation. The positions of the active site, ABE1, ABE2, and the Na^+ binding site are identified. The inhibitor is rendered in *red sticks*

F12 facilitates the release of the proteinase from the membrane surface at which it was produced. This is not the case for mIIa in which the proteinase domain is in covalent linkage with the F12 region. Consequently, despite being a proteinase variant of thrombin, mIIa can bind membranes with approximately the same affinity as does prothrombin allowing its accumulation on the membrane surface and its ability to cleave substrates in a membrane-dependent fashion [28]. Membrane binding by mIIa and the presence of the covalently bound F12 region are likely responsible for its apparently different sub-set of activities in comparison to thrombin.

Specificity of Prothrombinase for Prothrombin

Because of the interpretation problems posed by the sequential enzyme-catalyzed reactions in thrombin formation, mechanistic insights awaited the development of a series of recombinant substrate forms and isolated intermediates to allow studies of all possible four cleavage reactions. Early studies focused on using P2 in the absence of F12 as the simplest possible substrate for prothrombinase with a single cleavage site and lack of complexity arising from its ability to bind membranes [29]. Even these initial approaches provided evidence for a drastic difference in the mechanisms underlying protein substrate recognition in comparison to the specific binding and cleavage of peptidyl substrates that provided the formalisms for considering the mechanistic details of prothrombinase function until then. Short peptidyl substrates interact in a very limited way with the active site of Xa within prothrombinase to form a Michaelis complex prior to their cleavage and product release. A hallmark of this mechanism is the observation of competitive inhibition by reversible inhibitors that target the active site of factor Xa [29]. In contrast, the same inhibitors were found to be classical noncompetitive inhibitors of P2 cleavage by prothrombinase, implying that occluding the active site has no effect on the affinity of the protein substrate for prothrombinase. This can be accommodated by a multistep model for protein substrate binding in which the initial interaction between the substrate and prothrombinase occurs at extended surfaces on the enzyme (exosites) distant from the active site, followed by its active site docking and catalysis before product release (Fig. 4.6). Exosite binding is the main determinant for substrate affinity while the active site

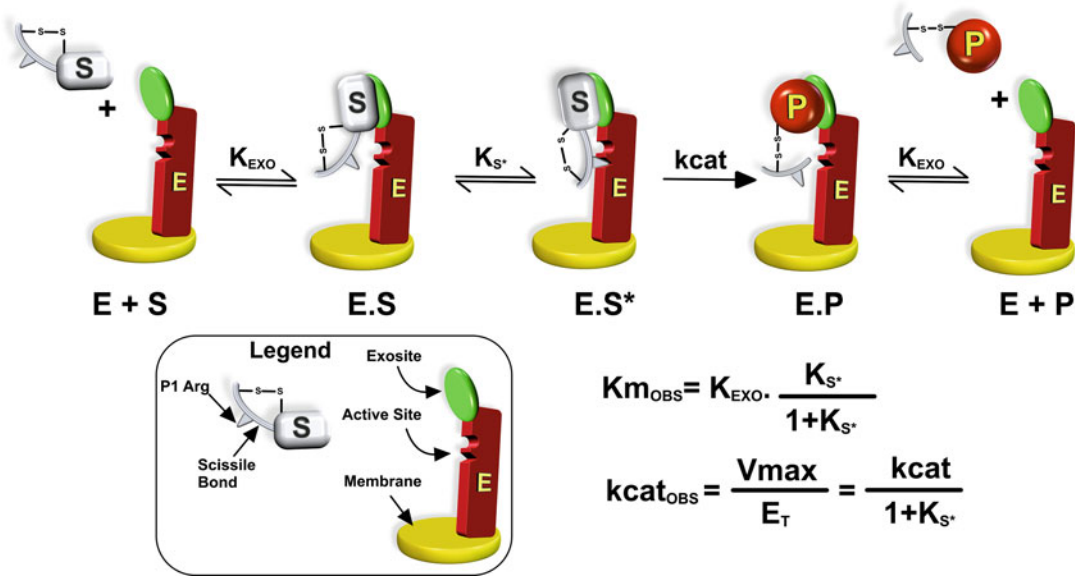

Fig. 4.6 The dock and lock pathway for protein substrate recognition by prothrombinase. The scheme illustrates the pathway for the recognition and cleavage of P2 by prothrombinase. The initial binding interaction between substrate (S) and prothrombinase (E) to form ES results from exosite-dependent interactions which leaves the active site unligated. Exosite binding is followed by a unimolecular binding step in which structures flanking the cleavage site engage the active site of the enzyme before catalysis can occur. The product (P) is also bound to E by exosite interactions before it is released. The graphic legend highlights the important features of S and E. The composite nature of the steady state kinetic constants is illustrated by derivation employing the rapid equilibrium assumption. Ks* is defined as [E.S]/[E.S*]. Taken with permission from reference [65]

docking step occurs in a unimolecular step before cleavage and contributes to the perceived rate constant for catalysis. The resultant composite nature of the steady state kinetic constants (Fig. 4.6), that derive from this mechanism illustrate how the active site docking step influences the observed rate constant for catalysis. This suggests that large improvements in $kcat_{obs}$ can result from simply modulating active site binding to prothrombinase and likely represents a component mechanism of how factor Va functions as a cofactor within prothrombinase.

An important implication of this observation is that the sequence-specific cleavage of prothrombin by prothrombinase is enforced by the exosite-mediated presentation of the scissile peptide bond to the active site of Xa within the complex rather than simply docking of the Glu/Asp-Gly-Arg sequence preceding the two scissile bonds with the active site. This idea has been tested with recombinant substrate variants in which residues before the scissile peptide bond were replaced with those found in other coagulation zymogens not ordinarily cleaved by prothrombinase [30]. Prothrombinase efficiently cleaved these prothrombin variants with a similar apparent affinity but with a modest change in the rate of catalysis. Thus, in contrast to prevailing ideas in the field, the active site of Xa within prothrombinase can accommodate and cleave a wide range of peptidyl sequences provided the protein substrate can engage the enzyme through exosite binding and facilitate their presentation for active site docking. In an unexpected twist, the geometric constraints enforced by exosite binding were also not found to be absolute as prothrombinase was surprisingly tolerant of an N-terminal shift of the cleavage site by one or two residues with cleavage being lost at greater shifts [31]. This suggests some flexibility in the polypeptide tether linking the exosite binding region and sequences flanking the cleavage site that engage the active site of the catalyst in the second step.

How Ordered Cleavage of Prothrombin is Achieved

A suite of recombinant mutants of full length prothrombin and the isolated intermediates allowed similar studies for each of the four possible reactions of prothrombin activation even in the presence of membrane binding (Fig. 4.7). Each possible half reaction exhibited the same dock and lock behavior initially noted with P2 and provided insights into why prothrombinase acts on prothrombin in an ordered fashion. The substrates for each of the four half reactions were cleaved with equal affinities [22]. Even though the II_{QQ} variant is uncleavable, it also bound prothrombinase with the same affinity as the other substrate forms. This is consistent with a primary role for exosite tethering rather than active site docking in determining binding affinity. Preferential cleavage of prothrombin at Arg^{320} first followed by cleavage at Arg^{271} was evident from equal values of $kcat_{obs}$ for three of the four half reactions while $kcat_{obs}$ was decreased 30–40 fold for cleavage at Arg^{271} in II_{Q320}. These findings explain why 97 % or more of the flux towards thrombin formation catalyzed by prothrombinase would occur via the formation of meizothrombin. When interpreted within the context of the composite nature of $kcat_{obs}$ for the dock and lock mechanism of substrate recognition (Fig. 4.6), the findings imply that when prothrombin binds prothrombinase, Arg^{320} readily engages the active site while Arg^{271} does not. Defective active site engagement by Arg^{271} is corrected by prior cleavage at Arg^{320} to form mIIa. Indeed, these ideas have been independently confirmed by binding studies of the individual cleavage sites in this suite of prothrombin variants to engage the active site of Xa within prothrombinase [32].

Ordered Prothrombin Cleavage and the Zymogen to Proteinase Transition

The ordered action of prothrombinase on prothrombin has its roots in the constraints arising from a single kind of exosite tethering interaction

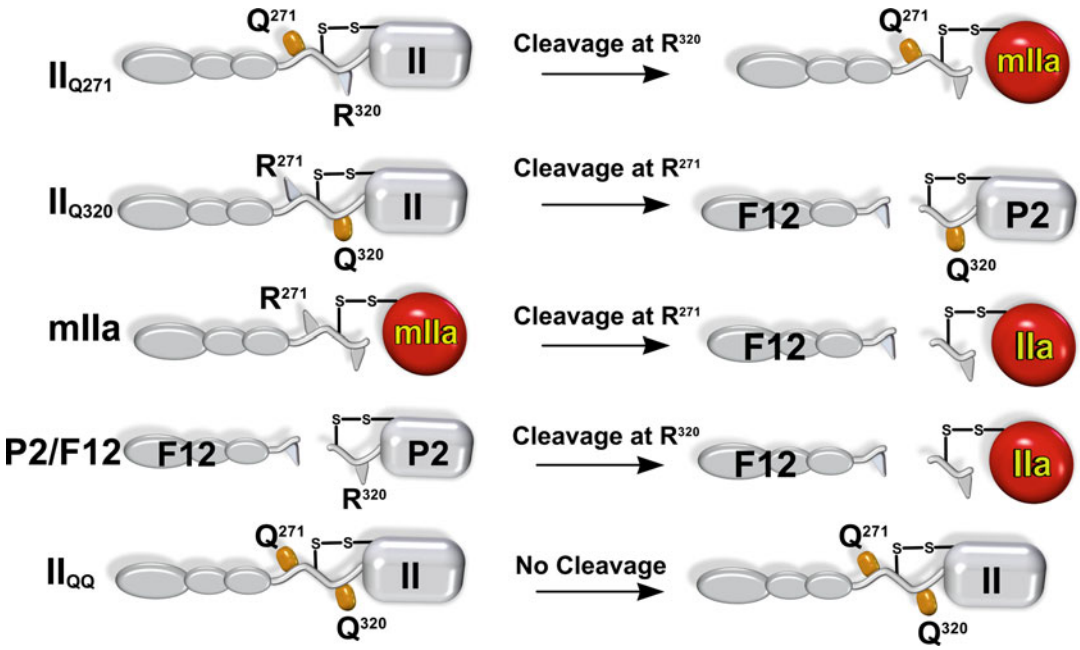

Fig. 4.7 Prothrombin variants necessary to resolve the kinetics of all possible steps of prothrombin cleavage. The cleavage reaction measured using the different substrate variants are indicated with the appropriate product either without prior cleavage (by substitution of one Arg with Gln) or following prior cleavage at one site. Taken with permission from reference [65]

between substrate and enzyme that is responsible for the presentation of two distant sites, expected to be separated by ~30Å, for active site docking and cleavage. The second major contributor is the well-established conformational transitions that accompany the conversion of zymogen to proteinase following cleavage at Arg^{320} to form mIIa [33]. Geometric constraints imposed by exosite tethering allow Arg^{320} to readily dock at the active site of Xa within prothrombinase while the distant Arg^{271} site cannot (Fig. 4.8a). When the proteinase is formed by prior cleavage at Arg^{320}, the comparably tethered mIIa substrate allows the previously distant Arg^{271} site into the vicinity of the active site for docking and cleavage (Fig. 4.8b). These concepts underlying the sequential presentation of the two sites to prothrombinase can be further illustrated by the manipulation of prothrombin between zymogen-like and proteinase-like forms. By exploiting the sequence-specific nature of the molecular sexuality process in proteinase formation, prothrombin variants were created that could be cleaved normally at Arg^{320} but failed to undergo maturation to proteinase. Despite prior cleavage at Arg^{320} the resultant mIIa was very poorly processed at Arg^{271} and persisted as an intermediate (Fig. 4.8c). Conversely, covalent stabilization of uncleaved prothrombin in a proteinase-like conformation enhanced its cleavage at Arg^{271} with a concomitant decrease in the rate of cleavage at Arg^{320} (Fig. 4.8d). This summarizes the essential role of exosite-dependent

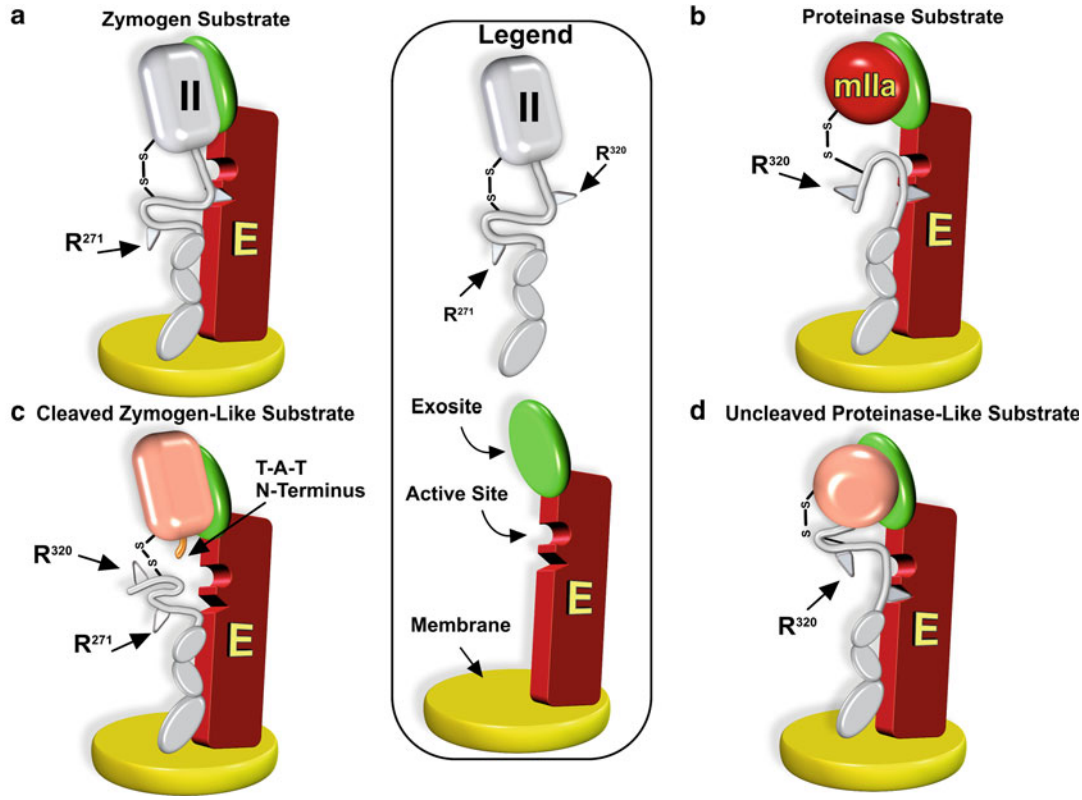

Fig. 4.8 Active site docking by the two substrate sites is driven by the zymogen or proteinase-like character of exosite-bound substrate. Exosite-binding constrains substrate presentation such that when the substrate is the zymogen, Arg^{320} preferentially engages the active site for cleavage (*Panel A*). Conversely, the Arg^{271} site readily engages the active site when the substrate is the proteinase (*Panel B*). Prior cleavage at Arg^{320} in a variant that remains zymogen-like yields impaired cleavage at Arg^{271} (*Panel C*). Conversely conformational activation and stabilization of uncleaved prothrombin in a proteinase-like state yields increased cleavage at Arg^{271} at the expense of cleavage at Arg^{320} (*Panel D*). Taken with permission from reference [65]

substrate tethering and the geometric constraints imposed by the zymogen to proteinase transition in regulating prothrombin cleavage.

Protein–Protein and Protein–Membrane Interactions in Exosite-Tethering

Evidence points to a significant role for surfaces in the catalytic domain of factor Xa, removed from the active site, in contributing to the substrate binding exosite [34, 35]. This can be deduced from studies with nematode anticoagulant peptide C2 and a monoclonal antibody that bind Xa within prothrombinase and act as competitive inhibitors of protein substrate cleavage without engaging the active site or affecting active site function of prothrombinase. It also follows that exosite-dependent substrate tethering must be mediated by substrate structures distinct from residues flanking the two scissile bonds. Indeed, a fragment from the C-terminus of the proteinase domain within prothrombin, physically separable from substrate structures bearing the cleavage sites, can act as a classical competitive inhibitor of protein substrate cleavage [36]. Such inhibition is also achieved without affecting active site function within prothrombinase. Finally, considerable evidence has accumulated over the years implicating an important functional role for interactions between prothrombin and its derivatives and factor Va within prothrombinase [37–40]. While some of these observed interactions may be most relevant for the activation of factor V, probes that inhibit the binding of prothrombin derivatives to factor Va also inhibit protein substrate cleavage. Thus, it is likely that exosite binding results from interactions between the substrate and extended surfaces in both factors Va and Xa within prothrombinase. Inhibition of substrate binding can be accomplished by occluding either component interaction. This scenario implies that the exosite mediated dock and lock strategy for protein substrate recognition by prothrombinase may not apply to the function of Xa in the absence of Va. Thus, a large fraction of the rate enhancing effects of the cofactor probably derive from a change in the mechanistic strategy by which an otherwise poor substrate for active engagement is now effectively recognized and cleaved by prothrombinase. This conclusion is fundamentally different from the ideas that have prevailed until recently and could probably never have been reached without the kind of detailed mechanistic understanding developed over the years.

Because membrane binding is mediated by N-terminal fragment 1 region of prothrombin distant from the cleavage sites, it falls under the definition of an exosite interaction that could play an important role in tethering the substrate to prothrombinase. If so, altered exosite-dependent tethering because of suboptimal or defective interactions between the substrate and membrane would be expected to alter substrate affinity and the geometric constraints on the bound substrate that affect active site docking and the pathway for substrate cleavage. Both criteria are met when the four half-reactions of prothrombin activation are studied with the appropriate recombinant single cleavage site substrate variants but without γ-carboxyglutamate modifications to eliminate their interaction with membranes (Fig. 4.9) [11]. In this case, loss in geometric constraints is evident as a decrease in $kcat_{obs}$ for cleavage at Arg^{320} with a concomitant increase in $kcat_{obs}$ for cleavage at Arg^{271} in previously uncleaved prothrombin [11]. These reciprocal changes lead to a switch in the pathway for prothrombin cleavage from one that is essentially entirely via the formation of mIIa in the case of the membrane binding variant to one in which the flux to thrombin formation is ~80 % via the formation of P2. The most surprising result from these compensating changes is a very modest three- to fourfold decrease in the rate of thrombin formation. This flies in the face of conventional wisdom in field for the past 40 years where large numbers of investigators have sought to correlate the progress of warfarin anticoagulation or bleeding with plasma levels of uncarboxylated prothrombin [41]. There is no arguing that warfarin is an anticoagulant; it is just the effects of warfarin on a reduced rate of thrombin formation are most likely exerted at the level of the other vitamin K dependent proteins and

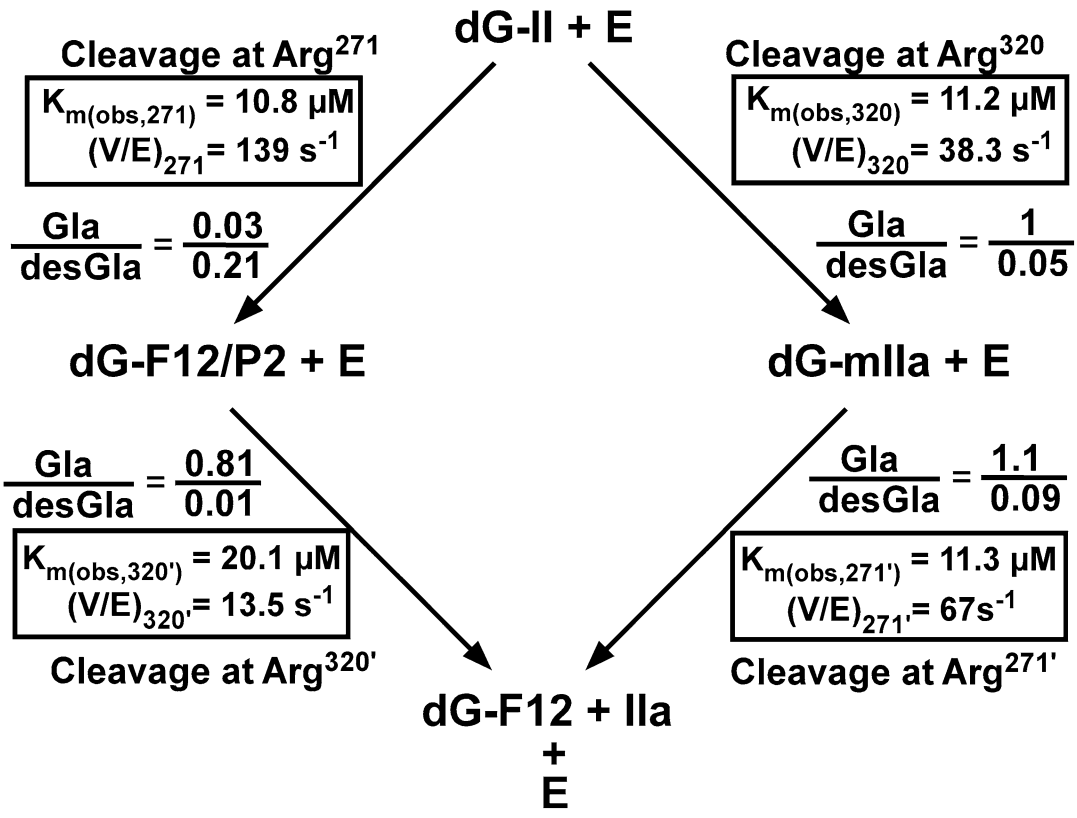

Fig. 4.9 Kinetics of cleavage of prothrombin with defective γ-carboxylation and membrane binding. The various des-gammacarboxy substrate forms are denoted as dG. Steady state kinetic constants for the individual steps are illustrated as are the rates for the individual steps at physiological concentrations of prothrombin for normal (Gla) and des-gammacarboxy (desGla) forms. E denoted prothrombinase

not on prothrombin. Thus, protein–protein and prothrombin–membrane interactions are component exosite binding interactions that work in concert to tether prothrombin to prothrombinase. Disrupting the membrane binding component is sufficient to relax the constrained presentation of the substrate to the enzyme and alter the pathway for thrombin formation (Fig. 4.10).

These concepts shed new light on the function of prothrombinase on natural membranes such as activated platelets or endothelial cells which are expected to expose limiting amounts of phosphatidylserine on their external leaflets [42]. Indeed, prothrombinase assembled on these cell surfaces forms thrombin by variable extents through the two possible activation pathways which can be replicated on synthetic membrane vesicles with low phosphatidylserine content [11]. The take home messages here are that prothrombinase function on physiologically relevant membrane surfaces can adequately described by biochemical mechanisms developed in purified and defined systems and that the amount of mIIa formed as an intermediate will be dependent on the cell type and its extent of activation in vivo.

Thrombin: The Multifunctional Proteinase

Interestingly, the exosite-dependent strategy employed by prothrombinase to achieve very narrow substrate specificity is also employed by thrombin to achieve pan-specificity in its myriad of roles as an effector and regulator of coagulation. This was initially evident from the ability to

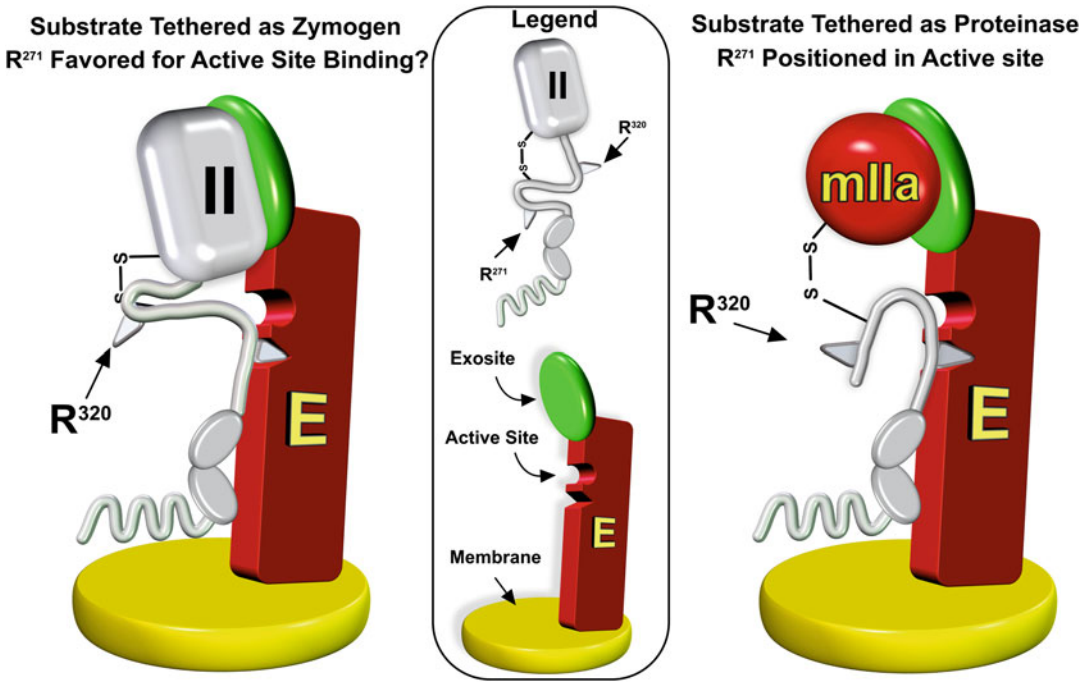

Fig. 4.10 Exosite-dependent tethering of substrate forms with defective γ-carboxylation to prothrombinase. The N-terminal Gla domain is shown as unfolded. In the enzyme-bound zymogen, loss of membrane binding now readily permits active site engagement by the Arg^{271} site. In the case of the previously cleaved proteinase intermediate (mIIa) membrane binding by the substrate has minimal impact on cleavage at the Arg^{271} site. The graphical legend highlights the salient features of the enzyme and substrate

greatly reduce its ability to clot fibrinogen by limited proteolytic cleavage but without impacting the ability of thrombin to cleave peptidyl substrates [43]. Subsequent and elegant studies of the inhibition of thrombin by the leech salivary inhibitor, hirudin, indicated that the femtomolar affinity for the interaction derived from bidentate binding with ABE1 and the catalytic site bridged by a polypeptide linker [44, 45]. The voluminous biochemical and structural studies that ensued have implicated an interaction motif with variations in detail on this theme, in explaining the binding and cleavage of a wide range of substrates for thrombin [46]. Thus, binding to ABE1 facilitates the engagement of a wide range of cleavage sites to the active site of the enzyme leading to cleavage, despite the fact that the residues flanking the site of cleavage reveal no compelling basis for the cleavage specificity of thrombin. On the other hand, ligation of ABE2 is mostly relegated to regulatory ligands such as heparin, which enhances the inactivation of thrombin by antithrombin III, the F2 domain of the F12 propiece, the γ′ region of fibrinogen, and glycoprotein Ib-IX-V [46, 47].

Thrombin also binds thrombomodulin through ABE1 which partly accounts for the procoagulant to anticoagulant switch in its specificity essential for the regulation of coagulation [48]. The binding of thrombin to the cofactor allows it to acquire the ability to activate protein C with a concomitant loss in its ability to cleave most other substrates. The occlusion of ABE1 by thrombomodulin prevents substrate binding by a host of its procoagulant substrates that utilize ABE1, thereby leading to a loss in the ability of thrombin to cleave these substrates [49, 50]. The structure of thrombin bound to a thrombomodulin fragment initially suggested that thrombomodulin would provide an additional surface to which protein C could bind therefore allowing for its cleavage and the anticoagulant function of

activated protein C. Subsequent studies unexpectedly failed to reveal evidence for this newly acquired exosite-dependent substrate recognition mechanism by the thrombin–thrombomodulin complex and instead point to a change in the way the scissile bond in protein C engages the active site of thrombin in complex with thrombomodulin [51]. This idea also likely applies to the ability of thrombomodulin-bound thrombin to activate TAFI and inhibit fibrinolysis [52].

Thrombin Allostery . A second aspect of the switch in specificity between procoagulant and anticoagulant functions of thrombin lies in the concept of proteinase allostery initially proposed as the idea that thrombomodulin likely induces a conformational change in the active site of thrombin to allow the acquisition of protein C activation function [53]. This idea is varyingly supported by a series of studies although it is not obvious from the X-ray structure of the thrombomodulin–thrombin complex [48]. Nevertheless, proteinase allostery originating from the ligation of ABE1 or other sites on the proteinase is likely an important feature underlying the many functions of thrombin that is supported by thermodynamic and NMR studies [54, 55].

A voluminous literature employing mutagenesis, functional and structural studies has accumulated in the past 20 years dealing with the subject of thrombin allostery. A good fraction of it deals with the binding of Na^+ to thrombin to affect its partitioning between a "Slow" form with preferential anticoagulant function and a "Fast" form with preferential procoagulant function. These allosteric changes are proposed to arise from linkage effects grounded in the idea that thrombin is only 50 % saturated with Na^+ in the physiological milieu [56, 57]. More recent measurements at physiological pH indicate a greater binding affinity for Na^+ than initially surmised from studies at pH 8.0 and indicate that thrombin would be near saturably bound to Na^+ in blood [54]. The physiological import of thrombin allostery arising from Na^+-binding and dissociation is now called into question regardless of the number of papers published on the subject.

Primarily as a result of mutagenesis studies and x-ray crystallography, the ideas of Fast (Na^+ bound) versus Slow (Na^+ free) thrombin have given way to a discussion of E* and E states also ascribed with different functions but variously stabilized by a bewildering array of mutations including those in highly conserved disulfide bonds [57, 58]. Accordingly, various thrombin mutants have been prepared with enhanced or preferential anticoagulant function with associated impairment in procoagulant function [59]. While these findings clearly indicate the ability to somehow dissociate the two opposing functions of thrombin, a coherent picture of how this can be achieved in multiple ways, within the context of E and E* thrombins, fails to emerge. This is particularly evident in the proposal that an uncleaved zymogen precursor of thrombin has been ascribed the properties of the E* form.

Is Thrombin Always a Proteinase?

Because proteinase formation results from irreversible cleavage at Arg^{15c} it is implicitly assumed that cleavage irreversibly stabilizes the proteinase. In contrast, early studies with chymotrypsin documented the existence of interconverting zymogen-like and proteinase-like species at near-neutral pH attributable to the protonation or deprotonation of Ile^{16c} [60]. Another notable exception is factor VIIa, which exhibits the characteristics of a zymogen until it binds its cofactor, tissue factor [61]. Investigation along these lines was prompted by the findings that the F12 pro-piece, produced during thrombin formation, bound the zymogen precursor P2 with substantially improved thermodynamics and affinity in comparison to thrombin [27]. In contrast, ligands for ABE1 such as thrombomodulin and hirugen bind thrombin more favorably than the zymogen P2 [38]. Thermodynamic studies were pursued using thrombin and P2 to define the limits of zymogen and proteinase along with a series of mutant variants variably stabilized along the pathway to proteinase formation [54]. The binding isotherms showed large and compensating enthalpic (ΔH) and entropic (ΔS) differences in the binding of F12 to these various forms depending on their increasing proteinase-like character.

As such enthalpy/entropy compensation is expected for ligand to related states that are in reversible equilibrium with each other, the findings surprisingly indicate that zymogen, zymogen-like, proteinase-like and proteinase states can reversibly interconvert along this thermodynamic trajectory. Accordingly, a strong active-site binding ligand could drag the zymogen-like form of thrombin along this thermodynamic trajectory to resemble the proteinase-like form. Conversely, removal of Na^+ while keeping the ionic strength constant could bring back the proteinase along the same trajectory to possess the thermodynamic properties of a zymogen-like form.

These unexpected and new ideas, grounded in thermodynamic measurements, suggest that thrombin can reversibly distribute along a continuum of zymogen-like and proteinase-like states even after cleavage but dependent on the complement of ligands that are bound to it (Fig. 4.11). This reversible distribution lies in the thermodynamically favorable ligation of ABE2 by F12 in the zymogen-like over the proteinase-like form and the converse being true for active site ligands or ligands for ABE1 (Fig. 4.11). Despite its dubious regulatory significance, Na^+-binding is a useful tool to manipulate this equilibrium as it is part of the folded structure of the proteinase but does not bind the zymogen.

These ideas provide a new formal framework for the consideration of allostery in thrombin and perhaps some of the other coagulation proteinases. Clearly, zymogen-like and proteinase-like

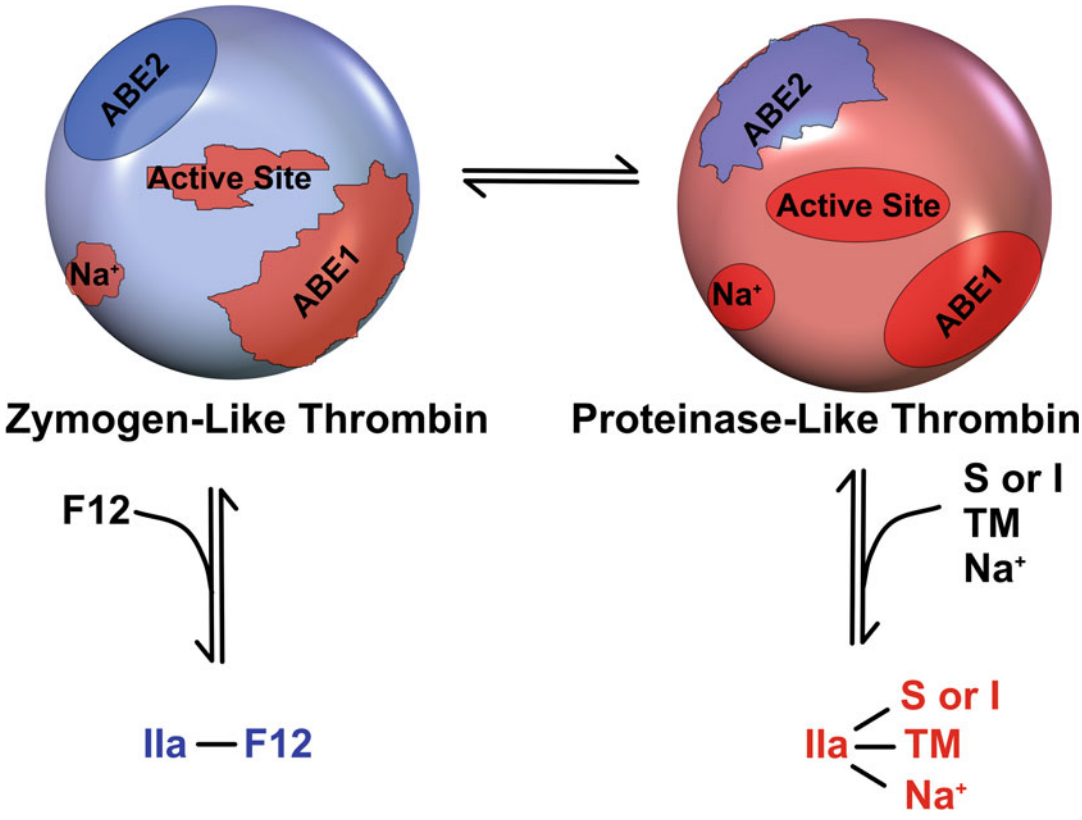

Fig. 4.11 Reversible interconversion of thrombin between zymogen-like and proteinase-like states. In the zymogen-like form, ABE1, the active site and the Na^+ site are illustrated as distorted while ABE2 is optimally functional. In contrast, ABE2 is distorted in the proteinase-like form while the other sites are optimally formed. F12 binds ABE2, while thrombomodulin (TM) binds ABE1. The active site can be ligated by substrates (S) or inhibitors (I). Taken with permission from reference [65]

forms will exhibit variable catalytic function which can be regulated by ligand binding. This framework also provides an explanation for how a myriad of different mutations can seemingly dissociate the opposing functions of thrombin. Any destabilizing mutation will favor zymogen-like forms with reduced activity that can variably be rescued by ABE1-binding ligands depending on their concentration and affinity. As fibrinogen binding is relatively weak, its ability to rescue these destabilized forms will be commensurately weak leading to poor procoagulant function. Conversely, thrombomodulin binds with high affinity to ABE1 and will be far more effective in rescuing protein C activation in the destabilized mutants. This proposal is in line with the distorted structures seen for many of the selectively anticoagulant variants of thrombin [55].

Are Zymogen-Like Forms Biologically Meaningful?

Despite the ability to rationalize thrombin allostery in terms of the ligand-dependent reversible interconversion between zymogen-like and proteinase like forms, there is doubt whether this can be biologically meaningful. This uncertainty arises from the weak interaction between F12 and thrombin and the fact that its binding to ABE2 and favor zymogen-like forms lies at the heart of the ligand-dependent shuttling of thrombin between the two proposed states (Fig. 4.11). Given the relatively weak binding of F12 to thrombin, a significant fraction of the enzyme is unlikely to be regulated in this way [54]. Reversible binding is not an issue in the case of mIIa in which the F12 region is covalently bound to the proteinase domain (Fig. 4.4). The associated prediction that mIIa would be expected to be exceptionally zymogen-like was tested and confirmed in rapid kinetic studies of ligand binding to its active site [62]. These studies revealed the slow equilibration of mIIa between equally populated zymogen-like and proteinase-like states [62]. In accordance with the predictions from studies with thrombin, the distribution between the two forms could be altered in both directions by adding thrombomodulin or by removing Na^+. In rapid kinetic studies using excess prothrombinase to affect near instant cleavage at Arg^{320} by prothrombinase, the zymogen-like form of mIIa was initially produced that only slowly equilibrated with the proteinase-like species (Fig. 4.12). This slow and reversible equilibrium represents the rate-limiting step in thrombin formation because cleavage at Arg^{271} to convert mIIa to thrombin requires its stabilization in the proteinase-like configuration (Fig. 4.8). Consequently, mIIa accumulates in vast excess over levels that would be predicted from the steady state kinetic constants for the sequential cleavage reactions. This represents a major advance in the understanding to prothrombin activation by prothrombinase. The biological significance of this finding lies in the slow equilibration between zymogen-like and proteinase-like forms and the fact that only the proteinase-like form can be processed further to thrombin. The zymogen-like mIIa will be refractory to inhibition by Serpins and be readily available to be washed downstream from its site of vascular damage. Given the fact that ABE1 ligands, such as thrombomodulin, can rescue variants with zymogen-like character, the ability of zymogen-like mIIa to be rescued in this manner and participate in protein C activation may be an important regulatory process in limiting expansive clot growth following vascular damage.

Conclusions

Despite their structural similarity, the proteinases of blood coagulation act on their cognate macromolecular substrates with narrow and defined specificity. This is accomplished by employing a conserved architecture and similar strategies to achieve specificity and enhanced function requisite for a rapid and highly regulated clotting response. Many of these strategies are exemplified in the process of prothrombin activation and by the functions of the resultant thrombin. Mechanistic studies with prothrombinase and thrombin reveal important principles by which regulated function is achieved. These rely on the

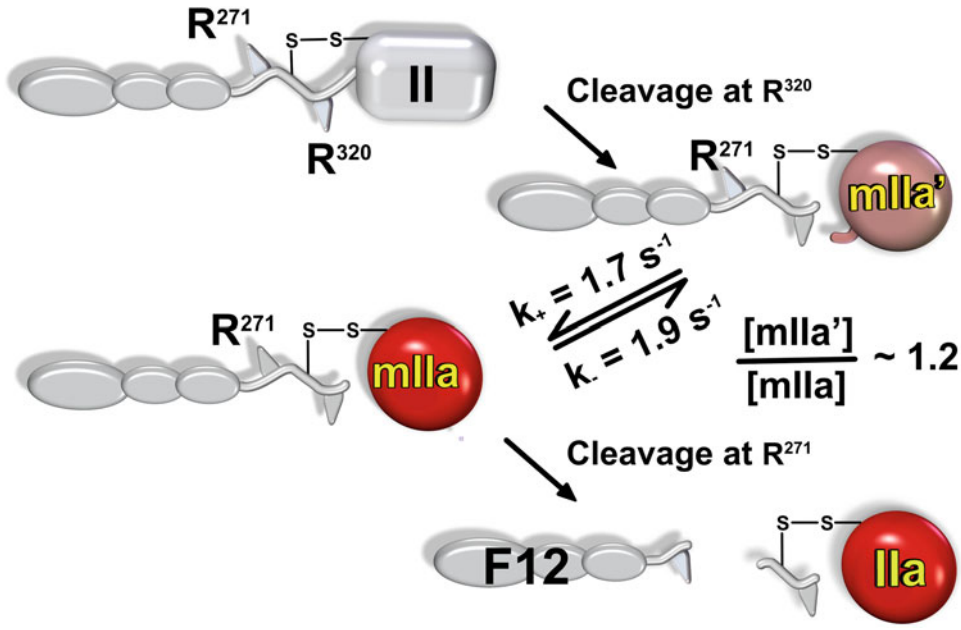

Fig. 4.12 A new rate-limiting step in thrombin formation. Cleavage of prothrombin by prothrombinase at Arg^{320} yields a slowly equilibrating mixture of zymogen-like meizothrombin (mIIa') and proteinase-like meizothrombin (mIIa) that are equally populated. Only the proteinase-like population of mIIa is further processed at Arg^{271} to produce thrombin. This new equilibrium derives from the covalent linkage of the N-terminal F12 region with the catalytic domain. Taken with permission from reference [65]

use of exosite interactions to bind the substrate and achieve very narrow specificity in the case of prothrombinase or pan-specificity in the case of thrombin. The second major mechanistic insight relates to the unexpectedly important role of the zymogen to proteinase transition in regulating prothrombin cleavage and in the process of thrombin allostery affected by its various ligands. Elements of these strategies are evident in the function of the other coagulation enzymes suggesting that they may widely apply and eventually provide an explanation for the diverse functions of the homologous enzymes of coagulation.

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