Thrombin: Physiology and Disease

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Editors

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Physiology and Disease

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Preface

The elucidation of the coagulation cascade has been one of the major accomplishments of biomedical sciences in recent years. The central role of thrombin, the plethora of factors and the multiple control mechanisms involved in haemostasis have been well characterized. However, new insights in the structure, functions and regulatory roles of thrombin in vascular physiology and development, in neuronal system, in tumor biology, tissue repair and angiogenesis have been gained by new powerful techniques and pioneering work of leading scientists.

In the chapters of this book it was attempted to provide a comprehensive present day view of thrombin, as an enzyme in relation to blood coagulation and in relation to its receptors. The effects of thrombin on various cell types and in patho-physiological conditions are discussed. This book is written to integrate the current understanding of thrombin basic mechanisms in vascular, inflammatory, neuronal and tumor cells and molecular biology with the experimental and clinical implications of these advances. Our goal was not to provide an exhaustively referenced compendium of the many topics that touch upon thrombin functions. Instead, we sought to create a practical and concise summary that would be equally useful for those first entering the field and for those with expertise in one facet of thrombin function wishing to learn more about others. Special emphasis was given to the new roles of thrombin and its receptors in vascular and tumor biology as well as angiogenesis, which present a challenge to translate this knowledge to therapeutic targets

We believe that this book will solidify the concept that thrombin and its receptors are a dynamic, vibrant field with clear implications for understanding the pathogenesis of several diseases (cardiovascular, neuronal, cancer) and for the treatment of patients. We hope that it will serve as a useful resource, not only for those involved in education and in the pursuit of new research programs, but also for those caring for patients with above diseases.

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Contents

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Chapter 1
Thrombin: Structure, Functions, and Regulation

Enrico Di Cera and Andras Gruber

Abstract Thrombin is a Na^+-activated, allosteric serine protease that plays opposing functional roles in blood coagulation. Binding of Na^+ is the major driving force behind the procoagulant, prothrombotic, and signaling functions of the enzyme, but is dispensable for cleavage of the anticoagulant protein C. The anticoagulant function of thrombin is under the allosteric control of the cofactor thrombomodulin. Recent structural advances have shed light on the remarkable molecular plasticity of this enzyme and the molecular underpinnings of thrombin allostery mediated by binding to exosite I and the Na^+ site. Thrombin exists in three forms – E*, E, and E:Na^+, which interconvert under the influence of ligand binding to distinct domains. The transition between the Na^+-free slow form E and the Na^+-bound fast form E:Na^+ involves the structure of the enzyme as a whole, and so does the interconversion between the two Na^+-free forms E* and E. E* is most likely an inactive form of thrombin, unable to interact with Na^+ and substrate.

1.1 Introduction

Thrombin is a serine protease of the chymotrypsin family, which includes enzymes involved in digestion and degradative processes, blood coagulation, cell-mediated immunity and cell death, complement, fibrinolysis, fertilization, and embryonic development. Once generated in the blood from its inactive precursor prothrombin, thrombin plays two important and paradoxically opposing functions (Di Cera 2008). It acts as a procoagulant factor when it converts fibrinogen into an insoluble fibrin clot that anchors platelets to the site of lesion and initiates processes of wound repair.

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This action is reinforced and amplified by activation of the transglutaminase factor XIII that covalently stabilizes the fibrin clot, the inhibition of fibrinolysis via activation of TAFI, and the proteolytic activation of factors V, VIII, and XI. In contrast, thrombin acts as an anticoagulant through activation of protein C. This function unfolds in vivo upon binding to thrombomodulin, a receptor on the membrane of endothelial cells. Binding of thrombomodulin suppresses the ability of thrombin to cleave fibrinogen and PAR_1, but enhances >1,000-fold the specificity of the enzyme toward the zymogen protein C. The reaction is further enhanced by the presence of a specific endothelial cell protein C receptor. Activated protein C (APC) cleaves and inactivates factors Va and VIIIa, two essential cofactors of coagulation factors Xa and IXa, which are required for thrombin generation, thereby downregulating both the amplification and progression of the coagulation cascade (Esmon 2003). Hijacking of thrombin by thrombomodulin and activation of protein C in the microcirculation constitute the natural anticoagulant pathway that prevents massive intravascular conversion of fibrinogen into an insoluble clot upon thrombin generation. In addition, thrombin is irreversibly inhibited at the active site by the serine protease inhibitor antithrombin with the assistance of heparin (Gettins 2002; Olson and Chuang 2002) and by the thrombin-specific heparin cofactor II (Tollefsen 2007). Important cellular effects are triggered by thrombin cleavage of protease-activated receptors (PARs) (Coughlin 2000), which are members of the G-protein-coupled receptor superfamily (Brass 2003). Four PARs have been identified, which share the same basic mechanism of activation: thrombin and other proteases cleave at a specific site within the extracellular N-terminus, exposing a new N-terminal tethered ligand domain that binds and activates the cleaved receptor (Coughlin 2000). Thrombin activation of PAR_1 (Vu et al. 1991), PAR_3 (Ishihara et al. 1997; Sambrano et al. 2001), and PAR_4 (Kahn et al. 1998; Xu et al. 1998; Nakanishi-Matsui et al. 2000) obeys this mechanism. PAR_1 is responsible for platelet activation in humans at low thrombin concentrations and its action is reinforced by PAR_4 at high enzyme concentrations (Coughlin 2000). Activation of PAR_1 and PAR_4 triggers platelet activation and aggregation and mediates the prothrombotic role of thrombin in the blood. PAR_3 is not present on human platelets, but is widely and abundantly expressed in other cell types. In the mouse, signaling in platelets is mediated entirely by PAR_4, with PAR_3 facilitating PAR_4 cleavage at low thrombin concentrations (Kahn et al. 1998; Nakanishi-Matsui et al. 2000). The efficiency of the coagulation cascade depends on the balance between the procoagulant and anticoagulant pathways. Thrombin is the key arbiter of this balance by virtue of its dual role and has therefore received utmost attention in structure–function studies and as a target of anticoagulant therapy (Bates and Weitz 2006).

1.2 Thrombin and Na^+

The most striking feature of thrombin is its ability to interact with Na^+ and the effect that Na^+ binding carries on physiologically important interactions with procoagulant (fibrinogen), prothrombotic (PARs), and anticoagulant (protein C) substrates

(Dang et al. 1995, 1997; Di Cera et al. 2007). Wells and Di Cera (1992) demonstrated that the Na^+ activation of thrombin is specific and allosteric, as expected for a Na^+-activated type II enzyme (Di Cera 2006). The property is shared with other clotting factors and proteases involved in immune response (Dang and Di Cera 1996; Krem and Di Cera 2001; Di Cera 2006). Na^+ binding converts thrombin from a low-activity slow (Na^+-free) form to a high-activity fast (Na^+-bound) form (Wells and Di Cera 1992). The slow and fast forms are significantly (2:3 ratio) populated under physiologic conditions because the K_d for Na^+ binding is 110 mM at 37°C (Wells and Di Cera 1992; Prasad et al. 2003; Bah et al. 2006) and the physiologic [NaCl] (140 mM) is not sufficient for saturation. Hence, the slow–fast equilibrium in vivo is optimally poised for allosteric regulation, and this is all the more significant in view of the fact that the procoagulant and anticoagulant activities of thrombin are partitioned between the fast and slow forms, respectively (Dang et al. 1995). Na^+ binding is required for optimal cleavage of fibrinogen and activation of factors V, VIII, and XI necessary for the explosive generation of thrombin in the coagulation cascade, but is dispensable for cleavage of protein C. This proves that Na^+ is the major driving force behind the procoagulant role of the enzyme in the blood (Di Cera et al. 2007; Di Cera 2008). Na^+ binding also promotes the prothrombotic and signaling functions of the enzyme by enhancing the cleavage of PAR1, PAR3, and PAR4. Owing to the allosteric nature of thrombin, any effect that destabilizes Na^+ binding stabilizes the slow form and produces an anticoagulant effect by prolonging the clotting time (reduced fibrinogen cleavage) and reducing platelet activation (reduced PAR1 cleavage). Indeed, several naturally occurring mutations of the prothrombin gene, such as prothrombin Frankfurt, Salakta, Greenville, Scranton, Copenhagen, and Saint Denis, affect residues linked to Na^+ binding (Pineda et al. 2004a) and are often associated with bleeding.

1.3 Thrombin Structure

Thrombin bears the chymotrypsin-like fold where two 6-stranded β-barrels come together asymmetrically to host at their interface the residues of the catalytic triad, H57, D102, and S195 (chymotrypsinogen numbering). Thrombin is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) residues that are covalently linked through a disulfide bond between residues C1 and C122 (Fig. 1.1). The standard "Bode" orientation puts the A chain in the back of the molecule, opposite to the front hemisphere of the B chain that hosts the entrance to the active site and all known functional epitopes of the enzyme (Pineda et al. 2004a). The A chain has received little attention in thrombin studies and is considered an appendage of the activation process from prothrombin. However, several naturally occurring mutations of prothrombin involve residues of the A chain and are associated with severe bleeding. The functional defects in prothrombins Denver (E8K and E14cK), Segovia (G14mR), and San Antonio (R15H) have been attributed to perturbation of the zymogen → enzyme conversion and processing by factor Xa, resulting in severe

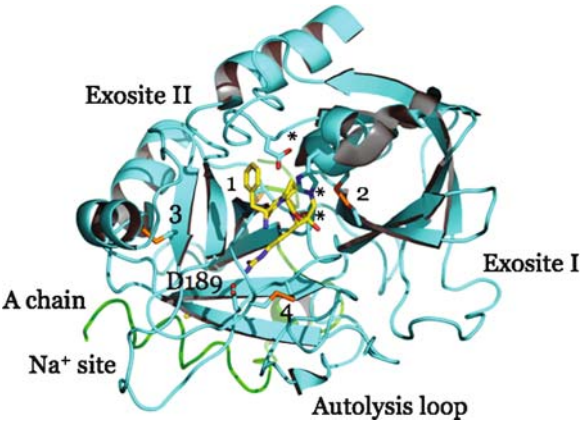

Fig. 1.1 Structure of thrombin bound to the active site inhibitor PPACK (stick model) and Na^+ (ball). The A chain runs in the back of the B chain. Disulfide bonds are numbered 1 (C1–C122), 2 (C48–C52), 3 (C168–C182), 4 (C191–C220). Relevant domains are noted. Catalytic residues (H57, D102, S195) are marked by asterisk, and D189 is labeled. The bound Na^+ is nestled between the 220-loop and the 186-loop and is within 5 Å from the side chain of D189. Numbering refers to chymotrypsin(ogen) (*see Color Plates*)

bleeding. Such explanation is obvious for the G14mR and R15H mutations that affect the P1 (R15) and P2 (G14m) sites of recognition by factor Xa, but not for the E8K and E14cK mutations of prothrombins Denver. Other naturally mutations, such as deletion of K9 or K10, are also associated with severe bleeding. Interestingly, the defect causes impaired fibrinogen and PAR_1 cleavage, reduced response to Na^+ activation and long-range perturbation of active site residues (De Cristofaro et al. 2006). The A chain is rich in charged residues that make polar interactions with partners of the B chain. Importantly, a significant number of negatively charged residues cluster toward the C-terminus, in close proximity to the Na^+ site. These charged residues may influence Na^+ binding or allosteric transduction, or both. The A chain is also optimally shaped to provide communication between the Na^+ site and the back of the active site region and could therefore influence substrate recognition and catalysis.

Trypsin-like specificity for Arg residues at P1 is conferred to thrombin by the presence of D189 in the S1 site occupying the bottom of the catalytic pocket. Thrombin has a preference for small and hydrophobic side chains at P2 that pack tightly against the hydrophobic wall of the S2 site defined by residues Y60a–P60b–P60c–W60d of the 60-loop. Residues at P3 point away from the thrombin surface, whereas aromatic and hydrophobic residues at P4 tend to fold back on the thrombin surface and engage the aryl binding site defined by L99, I174, and W215. The autolysis loop shapes the lower rim of access to the active site and contributes to the recognition of fibrinogen. The loop centered on K70 defines exosite I and is homologous to the Ca^{2+}-binding loop of trypsin and chymotrypsin. Exosite I contains several positively charged residues that give rise to an intense electrostatic field. The field

provides steering and optimal pre-orientation for fibrinogen, thrombomodulin, the natural inhibitor hirudin and PAR_1 to facilitate the formation of a productive complex upon binding. Structural and site-directed mutagenesis data support exosite I as a binding epitope for fibrinogen, fibrin, thrombomodulin, and the thrombin receptors PAR_1 and PAR_3 (Di Cera et al. 2007; Di Cera 2008). On the side of the enzyme opposite to exosite I, a C-terminal helix and its neighbor domains host a number of positively charged residues and define exosite II. This site is the locale for interaction with polyanionic ligands such as heparin and glucosaminoglycans. Heparin enhances inhibition of thrombin by antithrombin via a template mechanism in which a high-affinity heparin–antithrombin complex is first formed and then docked into exosite II and the thrombin active site by electrostatic coupling (Gettins 2002; Olson and Chuang 2002; Dementiev et al. 2004; Li et al. 2004). Exosite II is also the locale for thrombin interaction with the platelet receptor GpIb (De Cristofaro et al. 2000; Ramakrishnan et al. 2001; Celikel et al. 2003; Dumas et al. 2003).

The first X-ray structure of thrombin was solved in 1992 and revealed relevant information on the overall fold of the enzyme and especially on the arrangement of loops involved in macromolecular substrate recognition (Bode et al. 1992). The Na^+ binding site of thrombin was first identified crystallographically in 1995 from Rb^+ replacement (Di Cera et al. 1995). Na^+ binds 16–20 Å away from residues of the catalytic triad and within 5 Å from D189 in the S1 site, nestled between the 220- and 186-loops and coordinated octahedrally by two carbonyl O atoms from the protein (residues R221a and K224) and four buried water molecules. Structural biology has revealed important information on how thrombin utilizes both the active site and exosites for interaction with substrates, inhibitors, and effectors. Information on how thrombin recognizes substrate at the active site has come from the structure of the enzyme in complex with the irreversible active site inhibitor H-D-Phe-Pro-Arg-CH_2Cl (PPACK) (Bode et al. 1992). Arg at P1 ion-pairs to D189 in the S1 site; Pro at P2 fits snugly against P60b, P60c, and W60d in the S2 site; and Phe at P3, in the d-enantiomer, makes an edge-to-face interaction with W215 in the aryl binding site. The PPACK-inhibited structure reveals interactions that are relevant to recognition of natural substrates and confirms the key role played by the H-bonding network found within the active site of all trypsin-like enzymes bound to substrate (Hedstrom 2002). Crucial components of this network are the bidentate ion-pair between D189 and the guanidinium group of Arg at P1, the H-bonds of the carbonyl O atom of the P1 residue with the N atoms of G193 and S195 forming the oxyanion hole, the H-bond between the N atom of the P1 residue and the carbonyl O atom of S214, and the H-bonds between the backbone O and N atoms of the P3 residue with the N and O atoms of G216. This important arrangement of H-bonds has been documented in the structures of thrombin bound to fragments of the natural substrates fibrinogen (Stubbs et al. 1992), PAR4 (Bah et al. 2007), and factor XIII (Sadasivan and Yee 2000). The structure of thrombin in complex with the potent natural inhibitor hirudin has revealed how thrombin recognizes ligands at exosite I (Rydel et al. 1991). Hirudin blocks access to the active site of thrombin using its compact N-terminal domain and binds to exosite I via its extended, acidic C-terminal domain. The mode of interaction of the C-terminal domain of hirudin has later been documented in the

structures of thrombin bound to hirugen (Vijayalakshmi et al. 1994), fibrinogen (Pechik et al. 2004, 2006), PAR_1 (Mathews et al. 1994; Gandhi et al. 2008), PAR_3 (Bah et al. 2007), thrombomodulin (Fuentes-Prior et al. 2000), and heparin cofactor II (Baglin et al. 2002). Finally, the role of exosite II has been documented eloquently in the structures of thrombin bound to heparin (Carter et al. 2005), the fibrinogen γ′ peptide (Pineda et al. 2007), and GpIb (Celikel et al. 2003; Dumas et al. 2003).

1.4 Kinetics of Na^+ Activation

The discovery of the Na^+ effect on thrombin has provided a coherent framework to understand the structure and function of the enzyme, rationalized the molecular origin of the defects associated with several naturally occurring mutations of the prothrombin gene and offered an effective strategy to engineer thrombin for optimal anticoagulant activity in vivo which may one day translate into new therapeutic tools. Na^+ binding to thrombin is linked to a significant increase in intrinsic fluorescence with an initial rapid phase (in the microsecond time scale) that cannot be resolved within the dead time (<0.5 ms) of the spectrometer, followed by a single exponential slow phase (in the microsecond time scale) with a k_{obs} that decreases as $[Na^+]$ increases (Bah et al. 2007). Scheme 1 shows the two-step mechanism of Na^+ binding to thrombin.

$$\mathrm{E}^* \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} \mathrm{E} \underset{k_A}{\overset{k_{-A}}{\leftrightarrows}} \mathrm{E{:}Na^+}$$

Scheme 1 The two-step mechanism of Na^+ binding to thrombin

Thrombin exists in equilibrium between two forms, E* and E, which interconvert with kinetic rate constants k_1 and k_{-1}. Of these forms, only E can interact with Na^+ with a rate constant k_A to populate E:Na^+, which may dissociate into the parent components with a rate constant k_{-A}. The fast phase is due to the E–E:Na^+ interconversion involving Na^+ binding/dissociation, and the slow phase is due to the E–E* interconversion that precedes Na^+ binding. The three species in Scheme 1 portray thrombin in the Na^+-free (E and E*) and Na^+-bound (E:Na^+) forms. E and E:Na^+ in Scheme 1 are the two active forms of thrombin that account for the dependence of k_{cat} on $[Na^+]$ and correspond to the slow (E) and fast (E:Na^+) forms originally defined by Wells and Di Cera (1992). E* is a form of thrombin that cannot interact with Na^+ and substrate.

Precise determinations of the value of K_A as a function of temperature enable dissection of the thermodynamic components of Na^+ binding (Bah et al. 2006). Binding of Na^+ is characterized by a large enthalpy change of −22 kcal mol^{-1} that is compensated by a large entropy loss of −64 cal $mol^{-1}K^{-1}$. As a result of the enthalpy–entropy energetic compensation, the binding affinity of Na^+ is relatively weak (K_d in the millimolar range), as seen for many other M^+-activated enzymes.

An important consequence of the large enthalpy change is that the value of K_A becomes only about 10 M^{-1} at 37°C, which implies that under physiologic conditions of temperature and [NaCl] = 140 mM, thrombin is only 60% bound to Na^+. The fraction of thrombin in the procoagulant E:Na^+ form is about 60%, and the anticoagulant form E accounts for about 40%. The temperature dependence also demonstrates that, of the two Na^+-free forms, E* represents <1% of the population of thrombin molecules at 37°C. Thrombin has a total of nine Trp residues located in the B chain anywhere from 13 to 35 Å from the bound Na^+ (Pineda et al. 2004a). Single-site Phe mutants of each of the nine Trp residues were used to identify fluorophores responsible for the spectral changes associated with Na^+ binding (Bah et al. 2006). The 10% total increase in fluorescence observed for wild type is retained by five Trp mutants, namely, W29F, W51F, W60dF, W96F, and W237F. Two mutants of thrombin, W148F and W207F, experience >30% loss in total fluorescence change upon Na^+ binding. On the other hand, W141F and W215F lose >70% of the total fluorescence change. The fast phase of fluorescence increase directly linked to the transition from E to E:Na^+ in Scheme 1 is affected in all Phe mutants, vouching for a global effect of Na^+ binding on thrombin structure. W141 and W215 make a large contribution to the fluorescence change induced by Na^+ binding, and their mutation to Phe abrogates the fast phase completely. This implies that the environments of W141 and W215 change in the E*–E conversion, and more drastically in the conversion of E–E:Na^+.

1.5 Structures of E*, E, and E:Na^+

Current structural information on the molecular basis of Na^+-dependent allostery accounts for many important functional differences between the E and E:Na^+ forms. However, the documented structural changes are limited and do not explain the full complexity of the allosteric transition captured by functional studies that vouch for a remarkable conformational transition that transduces Na^+ binding into a global, long-range perturbation of the enzyme. With this caveat in mind, we will now analyze the results of recent crystallographic studies of the three forms of thrombin – E*, E, and E:Na^+.

Structures of E and E:Na^+ are highly similar, with rms deviations of the Cα traces of only 0.38 Å. There are five main structural differences between the slow (E) and fast (E:Na^+) forms of thrombin: (1) the R187–D222 ion-pair, (2) orientation of D189 in the primary specificity pocket, (3) conformation of E192 at the entrance of the active site, (4) position of the catalytic S195, and (5) architecture of the water network spanning >20 Å from the Na^+ site to the active site. Much of the activating effect of Na^+ can be explained in terms of the more favorable orientation of D189 in the S1 site, that improves K_m, and of S195 in the active site, that improves k_{cat}. The most significant structural change between the slow and fast forms provides evidence of long-range communication between the Na^+ site and the active site. In the fast form, there is a network of 11 water molecules that connect through

H-bonds the bound Na^+ to the Oγ atom of S195, located >15 Å away. The network provides the long-range connectivity needed to allosterically communicate information from the Na^+ site to the active site S195 and to residues involved in substrate recognition, such as D189 and E192. In the slow form, only seven water molecules occupy positions in the network equivalent to those seen in the fast form and the connectivity is radically altered.

The available structures of the slow form E and fast form E:Na^+ account for some basic functional properties of the two allosteric conformations of thrombin and explain most of the kinetic features linked to Na^+ binding and allosteric activation. However, rapid kinetic measurements of Na^+ binding clearly demonstrate that E is in equilibrium with a third thrombin form, E*, that is unable to interact with Na^+ (Scheme 1). The structural nature of E* is of considerable interest. E* has been suggested to portray an "inactive" slow form, unable to bind Na^+ and substrate or inhibitors at the active site. That hypothesis has gained prominence recently in the context of structure of the thrombin mutant D102N. The overall fold of D102N is similar to that in wild type, with a backbone rms deviation of 0.77 Å, compared to the Na^+-bound fast form (Pineda et al. 2004a). The structure shows changes in the primary specificity pocket and in the Na^+ binding site that are unprecedented in thrombin and the entire realm of serine proteases. These changes provide a plausible interpretation of the peculiar properties of the E* form. The 215–219 β-strand collapses into the primary specificity pocket. The side chain of R221a, located >10 Å away from the site of mutation, rotates 95° and brings the guanidinium group in contact with D189 in the primary specificity pocket. Altogether, the drastic shifts of W215 and R221a produce a conformation of thrombin that is self-inhibited by the hydrophobic engagement of the 60-loop and active site H57 by Trp-215, and of the acidic moiety of D189 in the primary specificity pocket by R221a. It should be pointed out, however, that although this structure reveals drastic changes at the level of W215 and perturbation of W141, it fails to account for changes at other Trp residues that are detected by fluorescence measurements. The true extent of conformational perturbation accompanying Na^+ binding to thrombin may ultimately defy resolution by X-ray structural biology.

1.6 Thrombin Interaction with Protein C

Two well-documented pathways of allosteric regulation exist in thrombin: one involves the Na^+ site and the other involves exosite I. As we have seen, binding of Na^+ to thrombin enhances activity toward procoagulant and prothrombotic substrates such as fibrinogen and PARs, whereas binding of thrombomodulin to exosite I enhances activity toward the anticoagulant protein C. Thrombin is the only enzyme in the blood capable of activating protein C (Di Cera 2003). Activated protein C is a natural anticoagulant that inactivates factors Va and VIIIa with the assistance of protein S, thereby promoting the downregulation of the coagulation cascade. The interaction of thrombin with protein C has been studied in considerable

detail. Under physiologic concentrations of Ca^{2+}, thrombin has only marginal affinity for protein C in the absence of thrombomodulin. The presence of thrombomodulin increases the k_{cat}/K_m of thrombin for protein C >1,000-fold because of a tenfold decrease in K_m and a 100-fold increase in k_{cat} (Esmon et al. 1983). In contrast to such drastic functional effects, the structure of thrombin bound to a fragment of thrombomodulin at exosite I failed to reveal significant conformational changes in the active site or other regions of thrombin (Fuentes-Prior et al. 2000). Such changes might have been obliterated by the presence of the active site inhibitor used in the crystallization. A number of peptides targeting exosite I influence allosterically the active site of thrombin, bring about significant changes in activity and even substrate specificity. One of these peptides, hirugen, is derived from the C-terminal fragment of hirudin. The structure of thrombin bound to hirugen was solved with the active site free (Vijayalakshmi et al. 1994), but again failed to reveal any significant conformational changes as for the thrombomodulin-bound structure (Fuentes-Prior et al. 2000). Solution to this conundrum has come unexpectedly from studies of how thrombin recognizes the receptors PAR_1, PAR_3, and PAR_4 (Bah et al. 2007; Gandhi et al. 2008).

1.7 Thrombin Interaction with the PARs

The prothrombotic role of thrombin depends mainly on cleavage of protease-activated receptors (PARs), which are members of the G-protein-coupled receptor superfamily (Coughlin 2000; Brass 2003). The potent natural inhibitor hirudin targets exosite I via its extended, acidic C-terminal domain (Rydel et al. 1991). An hirudin-like acidic domain is present in PAR_1 and PAR_3 immediately downstream of the tethered ligand domain and has been invoked in the ability of these receptors to engage exosite I of thrombin (Vu et al. 1991; Kahn et al. 1998). Recognition of PAR_4 is less dependent on exosite binding and relies almost entirely on the active site moiety (Ayala et al. 2001), especially through a pair of Pro residues at the P2 and P4 positions (Cleary et al. 2002; Jacques and Kuliopulos 2003). In addition, PAR_4 constructs carrying an hirudin-like acidic domain fail to produce enhanced binding or catalytic processing (Jacques and Kuliopulos 2003), suggesting that PAR_4 folds into a conformation unable to bind to exosite I. The cofactor function of PAR_3 on PAR_4 cleavage and activation by thrombin in murine platelets (Kahn et al. 1998; Nakanishi-Matsui et al. 2000) implies binding of both PAR4 and the cleaved form of PAR_3 to thrombin to form a ternary complex. After cleavage of PAR_3, thrombin would remain bound to the receptor via exosite I. This complex would then engage PAR_4 for proteolytic activation optimized by an allosteric effect of PAR_3 bound to exosite I on the active site moiety. A similar mechanism likely exists in human platelets, where PAR_4 can be cofactored by other receptors (Kahn et al. 1998). The cofactor function of PAR_3 on PAR_4 cleavage and activation has many features in common with the interaction of thrombin with protein C that is cofactored by thrombomodulin. Structural studies of thrombin–PAR interactions should reveal important details on the allosteric mechanism underlying binding to exosite I.

Murine thrombin inactivated with the single site mutation S195A was crystallized in complex with the extracellular fragment of murine PAR_3 (Bah et al. 2007), ^{38}SFNGGPQNTFEEFPLS-DIE^{56}, corresponding to the sequence downstream of the cleavage site at K37 and containing the hirudin-like motif ^{47}FEEFP^{51} predicted to bind to exosite I (Nakanishi-Matsui et al. 2000; Jacques and Kuliopulos 2003). The PAR_3 fragment engages exosite I of murine thrombin in a number of well-defined interactions. Overall, the surface of interaction between thrombin and PAR_3 is composed of a hydrophobic patch surrounded by a periphery of polar/electrostatic contacts. The presence of PAR_3 bound to exosite I causes a rearrangement of the 60-loop lining the upper rim of the active site entrance. In the free enzyme the indole ring of W60d partially occludes access to the active site and restricts specificity toward physiologic substrates and inhibitors. When PAR_3 binds to exosite I, the 60-loop shifts 3.8 Å upward and causes a 180° flip of W60d around the Cβ–Cγ bond that projects the indole ring into the solvent and opens up the active site fully. Key to this allosteric effect is the ability of W60d to move like a flap and regulate substrate diffusion into the active site. The structural flexibility documented for this and other Trp residues of thrombin is in agreement with recent spectroscopic measurements (Bah et al. 2006).

The murine thrombin mutant S195A was also crystallized in complex with the extracellular fragment of murine PAR_4 (Bah et al. 2007), ^{51}KSSDKPNPR↓GYPGKFCANDSDTL-ELPASSQA^{81} (↓ = site of cleavage), containing the cleavage site at R59. Overall, the thrombin conformation is similar to that seen in the thrombin–PAR_3 complex (rmsd, 0.52 Å), but W60d retains its canonical orientation with the indole ring pointing down over the active site in contact with PAR_4. Of great importance are the contacts made by PAR_4 with the active site of thrombin. R59 penetrates the primary specificity pocket and engages several residues. Together, P56 and P58 at the P4 and P2 positions of substrate produce a clamp that latches PAR_4 onto the active site of thrombin utilizing a large hydrophobic surface from the aryl binding site on one side and the 60-loop on the opposite side of the catalytic H57. The conformation of the ^{56}PNPR^{59} sequence of PAR_4 is practically identical to that predicted by NMR studies of the human PAR_4 sequence ^{44}PAPR^{47} (Cleary et al. 2002) and supports the critical role of this Pro pair in thrombin recognition uncovered by functional studies (Jacques and Kuliopulos 2003). Downstream from the scissile bond, the P1′ residue G60 initiates a turn followed by a short helical segment stabilized by a H-bond between the O atom of P62 and the N atom of C66. Without P62, the fragment of PAR_4 would likely pursue the 30-loop and exosite I of thrombin. The helix turn stabilizes and redirects PAR_4 toward the autolysis loop right below the entrance to the active site. The mode of interaction of PAR_4 with thrombin calls for numerous interactions with the active site and the aryl binding site, leaving exosite I and the 30-loop free. Cleavage of PAR_4 does not require binding to exosite I of thrombin, in agreement with the findings of earlier mutagenesis studies (Nakanishi-Matsui et al. 2000; Jacques and Kuliopulos 2003). The strategy used by PAR_4 to contact the active site of thrombin in a way that avoids interaction with exosite I is highly relevant to the interaction of thrombin with protein C, for which no structural information is currently avail-

able. The obvious conclusion to be drawn from the structures of murine thrombin bound to PAR_3 and PAR_4 is that the cleaved form of PAR_3 bound to exosite I does not interfere with the binding of the intact PAR_4 to thrombin. The cleaved PAR_3 and intact PAR_4 fragments bind to thrombin without overlap and can generate a ternary complex where PAR_3 functions as a cofactor that allosterically optimizes PAR_4 cleavage (Fig. 1.2).

The structure of the thrombin–PAR_3 complex offers convincing evidence that the position of W60d and the 60-loop can be modified allosterically by binding to exosite I when the active site of thrombin is free. A recent structure of thrombin bound to PAR_1 provides crystallographic evidence of a much larger conformational change experienced by thrombin when exosite I is bound to a ligand (Gandhi et al. 2008). Human thrombin inactivated with the single site mutation D102N was crystallized in complex with the extracellular fragment of human PAR_1, ^{42}SFLLRNPNDKYEPFWEDEEKN^{62}, corresponding to the sequence downstream from the cleavage site at R41 and containing the hirudin-like motif ^{52}YEPFWE^{57} predicted to bind to exosite I (Vu et al. 1991; Coughlin 2000). The structure documents a large conformational change that propagates from F34 and R73 in exosite I, to W215 in the aryl binding site and R221a in the 220-loop located up to 28 Å away on the opposite side of the active site relative to exosite I. The peculiar self-inhibited E* conformation documented originally for D102N (Pineda et al. 2006) can therefore convert into a catalytically active conformation through a structural transition that can be traced to a set of residues organized in four layers. A first layer directly in contact with ligands recognizing exosite I (F34 and R73), a second

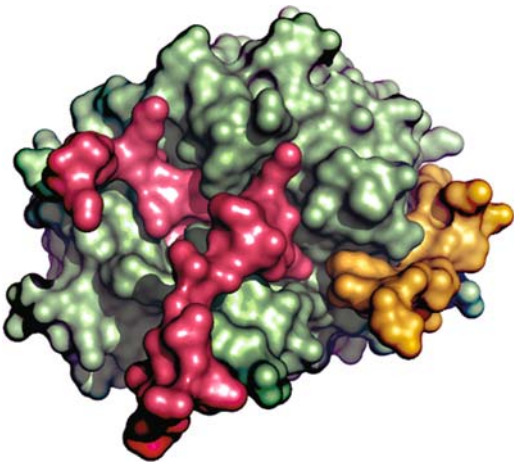

Fig. 1.2 Putative ternary complex of thrombin, cleaved PAR_3, and PAR_4 derived from an overlay of the crystal structures of the murine thrombin–PAR4 and thrombin–PAR_3 complexes. Thrombin refers to the thrombin–PAR_4 complex. Binding of cleaved PAR_3 to exosite I does not interfere with binding of PAR_4 to the active site of the enzyme. Cleaved PAR_3 may therefore act as a cofactor of PAR4 cleavage by thrombin (*see Color Plates*)

layer of "transducing" residues connecting to the 141–146 β-strand (M32 and Q151), a third layer comprising the interactions between the 141–146 and 191–193 β-strands (W141, N143, and E192), and a final layer where such interactions are transmitted to the 215–219 β-strand and the 220-loop via the C191:C220 disulfide bond and E146.

1.8 Dissociating Procoagulant and Anticoagulant Activities

The multifunctional nature of thrombin has long motivated interest in dissociating its procoagulant and anticoagulant activities. Thrombin mutants with anticoagulant activity help rationalize the phenotypes of several naturally occurring mutations and could eventually provide new tools for pharmacological intervention (Bates and Weitz 2006). A comprehensive library of Ala mutants was recently utilized to map the epitopes recognizing fibrinogen, protein C, and thrombomodulin in order to identify residues that contribute differentially to the procoagulant and anticoagulant functions of thrombin. Residues important for fibrinogen recognition are distributed over the entire surface of contact between enzyme and substrate and involve the 60-loop, exosite I, the primary specificity pocket, the aryl binding site, and the Na^+ binding site (Di Cera et al. 2007; Di Cera 2008). The surface of recognition between thrombin and protein C changes significantly upon thrombomodulin binding and is reduced mainly to the primary specificity pocket and portions of the 60-loop in the presence of cofactor. This makes it possible to identify residues that are significantly more important for fibrinogen recognition than protein C activation and to engineer thrombin mutants with enhanced anticoagulant activity in three steps: (1) identify residues that contribute differentially to fibrinogen and protein C recognition, (2) select among these residues those that make independent contributions to substrate recognition, and (3) construct multiple mutations involving these residues to achieve additivity. Targets can be set for optimal anticoagulant activity in vivo by demanding safety and potency of the constructs. For a mutant to be safe, activity toward fibrinogen should be <0.1% of that of wild type. Potency demands activity toward protein C to be >10% of that of wild type. These targets define a region in Fig. 1.3 where "ideal" anticoagulant thrombin mutants should map for selection. None of the 80 Ala mutants reported in Fig. 1.3 map in that region, but W215A and E217A stand out. Because W215 and E217 participate in fibrinogen recognition via distinct interactions, the double mutant W215A/E217A (WE) was constructed (Cantwell and Di Cera 2000) hoping for additivity of mutational effects that would boost the anticoagulant activity relative to the single mutations. Additivity was indeed found and WE mapped in the desired section in Fig. 1.3 (Cantwell and Di Cera 2000). The crystal structure of WE in the absence of inhibitors shows a conformation with the active site occluded by a collapse of the 215–217 strand, whereas the PPACK-bound form is similar to that of wild type (Pineda et al. 2004b). The mutant is practically inactive toward synthetic and natural substrates, and recovers activity only in the presence of thrombomodulin and protein

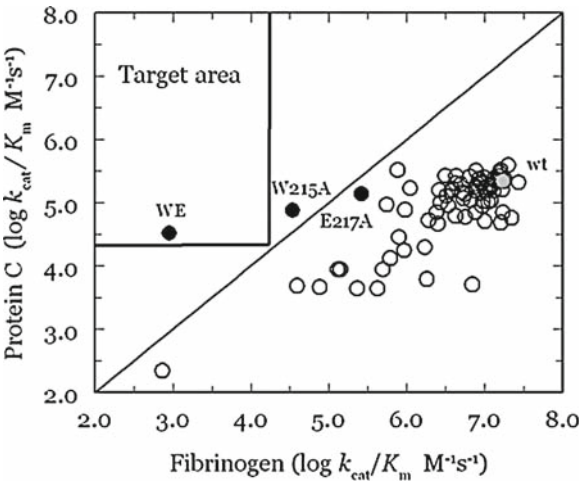

Fig. 1.3 Specificity constants k_{cat}/K_m for the hydrolysis of fibrinogen and protein C, in the presence of 10 nm thrombomodulin and 5 mm $CaCl_2$, under experimental conditions of 5 mm Tris, 0.1% PEG, 145 mm NaCl, pH 7.4, at 37°C. Plotted are the wild-type (*gray*) and 80 Ala mutants of thrombin. Safety and potency of an anticoagulant thrombin mutant for in vivo applications demand <0.1% activity toward fibrinogen and >10% activity toward protein C compared to wild type. These boundaries define a target region in the plot where mutations should map. W215A and E217A are the most promising single Ala mutants. Combination of the two mutations in the W215A/E217A produces additivity and a construct with the required anticoagulant profile

C. Hence, WE is presumably stabilized in the E* form and converts to the E form upon binding of thrombomodulin following a molecular transition similar to that documented for the interaction of the D102N mutant with PAR_1. These are the most desirable properties for a recombinant thrombin to be used in vivo as an anticoagulant.

1.9 WE: A Prototypic Anticoagulant/Antithrombotic Thrombin

Systemic administration of activated protein C (APC) has antithrombotic and anti-inflammatory effects that are now utilized in the treatment of severe sepsis (Bernard et al. 2001). Since infused thrombin activates protein C and APC is antithrombotic, thrombin infusion could act, in theory, as an antithrombotic agent. The thrombin analog WE was tested in a wide dose range for safety and efficacy in a baboon model of acute vascular graft thrombosis (Gruber et al. 2002, 2007). In this model, a permanent, surgically implanted arteriovenous shunt is temporarily extended with a thrombogenic vascular graft segment. Acute thrombus formation is visualized and quantified in real time using gamma camera imaging the deposition of radiolabeled platelets in the graft. WE was initially administered as an intravenous bolus in order

to evaluate pharmacokinetics. The lowest bolus dose of WE tested, 11 μg kg^{-1}, reduced platelet accumulation by 80% 1 h after the beginning of thrombosis, and was at least as effective as the direct administration of 40-fold more (0.45 mg bolus/kg) APC. Baboons treated with WE at doses as high as 200 μg kg^{-1} showed no clinical or laboratory signs of thrombosis, hemorrhage, or organ failure. No procoagulant activity could be detected for up to 1 week in baboon plasma obtained following bolus WE administration. Meanwhile, rapid systemic anticoagulation was observed, which dissipated with the biological half life of circulating APC, as determined in previous experiments in baboons. Higher doses of WE (>20 μg kg^{-1}) had pronounced anticoagulant effect, triggered by a burst in the levels of circulating endogenous APC following injection of WE (Gruber et al. 2006). High-dose WE infusion exhausted the cofactors of protein C activation before consumption of the substrate reserve and the process was rapidly downregulated >90% following a WE overdose. The exact molecular or cellular mechanism of this self-limiting pharmacological process is not yet known, but failure of protein C activation upon WE overdose can be overcome by cofactor supplementation using soluble recombinant thrombomodulin (Gruber et al. 2006). These results suggest that the thrombin–thrombomodulin complex is efficiently and rapidly inhibited in vivo and that thrombomodulin does not recycle rapidly once WE (and possibly thrombin) is bound to it. The true significance of this surprising finding is that pharmacological protein C activation by WE appears to be intrinsically safe, compared to all other accepted methods of anticoagulation, that are ultimately fatal when overdosed. Studies with low-dose WE infusions revealed that pharmacological levels of APC and marginal systemic anticoagulation could be maintained by continuous WE infusion for at least 5 h without substantial decrease in protein C levels. Since WE has potent antithrombotic effects even at very low doses that do not induce systemic anticoagulation (Gruber et al. 2007), natural protein C reserves and production could keep up with the consumption of protein C during sustained antithrombotic WE treatment.

The hemostatic safety of continuous WE infusion in comparison to equi-efficacious doses of low molecular weight heparin infusion for the prevention of acute vascular graft thrombus propagation in the baboon model was also evaluated. On the basis of previously established anticoagulant effect and antithrombotic efficacy of circulating APC in the baboon model, we originally predicted that, in systemic blood samples, an APTT prolongation of at least 1.5-fold over baseline, and APC levels at least 20-fold over baseline, sustained for at least 40 min, would result in a significant antithrombotic benefit. The results exceeded our expectations and we found that WE had potent antithrombotic effect even at a dose (2.1 μg kg^{-1} per 70 min) when the APTT was not demonstrably affected (Gruber et al. 2007). This indicated that WE was a very potent antithrombotic agent in primates (Gruber et al. 2007). Low doses of WE (2.1, 4.2, or 8.3 μg kg^{-1} per 70 min) outperformed higher doses of exogenous APC (28 and 222 μg kg^{-1} per 70 min) and were as efficient as interventional doses of intravenous enoxaparin (325–2,600 μg kg^{-1} per 70 min) in preventing the propagation of thrombi. The lowest dose of WE tested still increased circulating APC levels by approximately fivefold (to about 20 ng mL^{-1}), and we do

not yet know whether even lower doses of WE that may not detectably increase APC levels would also be efficacious. The comparably effective plasma concentration of exogenous (recombinant) APC exceeded the endogenous APC level following low-dose WE infusion by severalfold. The fact that WE is very effective at such small doses strongly argues for limited receptor-mediated mechanism. The considerably higher antithrombotic efficacy of WE, compared to exogenous APC, suggests that APC generated by WE on the surface of endothelial cells may remain transiently bound to the receptor and elicit additional effects by signaling via PAR_1 (Feistritzer et al. 2006; Gruber et al. 2007). WE may be retained on the cell surface in the vicinity of PAR_1 by receptors other than thrombomodulin, similar to the case of its interaction with platelet GpIb which appears to be active only under shear flow conditions (Berny et al. 2008).

The molar ratio of the equi-efficacious doses of WE and enoxaparin exceeded 1:1,000. However, this extraordinary potency would be ultimately and clinically irrelevant if the safety profile of WE were not substantially different from other anticoagulants. The striking finding was that these potent antithrombotic doses of WE infusion did not impair primary hemostasis, an outcome never before seen in baboons that received comparably effective doses of commercially available antithrombotic agents (Gruber et al. 2007). Interestingly, the comparably antithrombotic, and reasonably low doses of WT thrombin, 40 $\mu g\ kg^{-1}\ h^{-1}$, and WE, about 2 $\mu g\ kg^{-1}\ h^{-1}$, appear to be at least one order of magnitude apart to the advantage of WE, despite the 90% reduced activity of WE toward protein C. One of the possible explanations for this discrepancy could be the propensity of wild-type thrombin to bind to and activate abundant prothrombotic substrates (e.g., fibrinogen, PAR_1) in the blood flow, leaving only a fraction of the dose to diffuse across the boundary layer of flow to surfaces. Meanwhile, binding and interaction of WE with fibrinogen in blood are markedly reduced, the rate of such interactions as fibrinogen cleavage and inhibition by antithrombin are delayed by several orders of magnitude. The delay leaves more time for the administered enzyme to reach surfaces and become available for interaction with or consumption by transmembrane cofactors and receptors, such as thrombomodulin and PAR_1. This can thus lead to effective activation of the protein-C-dependent pathways on solid surfaces such as the endothelium. Altogether, this makes WE thrombin an agent that targets thrombosis and helps explain the very high antithrombotic efficacy of this enzyme.

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