Thrombin: multifunctional roles and therapeutic target

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Following vascular injury, blood loss is controlled by the mechanisms associated with haemostasis. During this process, the serine protease, thrombin, is generated locally at injured sites. Here it plays a pivotal role in both clot promotion and inhibition, as well as additional processes, such as cell signalling. Failure to tightly coordinated these functions can lead to either bleeding or thrombosis.

The crystal structures of thrombin, in combination with biochemical analyses of thrombin mutants, have provided insight into the ways in which thrombin functions and its activity is modulated. Due to its central role in haemostasis, thrombin remains a therapeutic target, as current antithrombotic therapies are invariably broad-spectrum and their use requires constant monitoring to minimise haemorrhagic risk. In this short review, we summarise recent progress in our understanding of the way in which this multifunctional proteinase interacts with its substrates during haemostasis.

**Thrombin generation**

Typically, haemostasis is a response to vascular injury. Vessel damage causes the cell surface receptor, tissue factor, which is normally present in extracellular locations, to become exposed to the blood [1, 2]. Tissue factor binds the plasma protease, factor VIIa. Once bound in this complex, factor VIIa proteolytically activates factor X. The inactive thrombin precursor, prothrombin, circulates in plasma at a concentration of ~1.4\(\mu\)M. Thrombin is generated through factor Xa activation of prothrombin. Cleavage first occurs at R320 to generate the intermediate, meizothrombin, and thereafter at R271 to generate thrombin [3]. This proteolytic activation liberates fragment F1+2, containing the prothrombin Gla and kringle domains. In prothrombin, these modules serve to localize prothrombin on platelet membrane surfaces as part of the activation (or “prothrombinase”) complex, that includes factor Va and factor Xa [Figure 1]. Once prothrombin is activated, thrombin can escape the prothrombinase complex and is then free to attack its target substrates [Figure 1]. Prothrombin is unique amongst the activated coagulation proteinases in that once activated to its serine proteinase derivative, thrombin, it loses the domains important for the initial recognition/activation interactions. Loss of these domains allows thrombin to diffuse freely to encounter, recognise, cleave and dissociate from its many substrates. Their loss also exposes cryptic functional regions [4], namely the active-site and the charged binding regions, termed exosites I and II, that are critical determinants in the specificity of thrombin [Figure 2].

**Thrombin structure**

The crystal structure of thrombin has revealed it to be highly homologous to other serine proteinases, such as chymotrypsin [5, 6]. A common feature of these enzymes is the presence of a serine residue (S195, chymotrypsin numbering) in the active-site cleft [Figure 2] that is involved in the nucleophilic attack of the target peptide bond. Thrombin has additional features that impart its specificity. Surface exposed loops and charged patches exist that surround the active-site. The 60- and γ-loops frame the active-site pocket containing the catalytic serine. The hydrophobic 60-loop is given structural rigidity by turns induced by two adjacent Pro residues (P60b, P60c). This loop interacts with hydrophobic residues on the amino-terminal side of the cleavage site of thrombin’s substrates. The more mobile hydrophilic γ-loop inter...
acts with substrate residues carboxy-terminal to the target scissile bond.

Thrombin has two anion-binding exosites (I and II) that are made up of clusters of surface exposed basic residues [Figure 2]. Exosite I is centred around residues R36, R71, R73, R75, Y76, R77a and K109/110 and exosite II includes residues R93, K236, K240, R101 and R233. These charged patches interact specifically with negatively-charged regions on thrombin cofactors and substrates. The importance and individual contribution to thrombin’s different functions has been reviewed extensively elsewhere.

Another important feature on thrombin is the loop containing a Na⁺-binding site [7]. The binding of a sodium ion allosterically modifies thrombin. Once bound, Na⁺ alters thrombin function, increasing access of small substrates to the active-site and favouring certain procoagulant substrates (i.e. fibrinogen and PAR1) over anticoagulant ones. The residues energetically linked to Na⁺-induced allosterie are D189, E217, D222 and Y225 [8]. Due to the Na⁺ concentration in blood (~140mM), this site is always occupied under normal physiological conditions. Mutants such as E217K and W215A/E217A exhibit greatly reduced procoagulant activities, but retain their anticoagulant function. Such mutants may represent potential therapeutic anticoagulants [9, 10].

**Haemostatic substrates of thrombin**

The primary function of thrombin is to catalyse the conversion of fibrinogen to fibrin. For this, two short fibrinopeptides (FPA ~ 16aa, FPB ~ 14aa) are cleaved from the Aα and Bβ chains, respectively. Cleavage of FPA occurs first to form a fibrin monomer (teemed fibrin I), which spontaneously polymerises to form protofibrils. Cleavage of FPB generates fibrin II protofibrils that undergo lateral aggregation. The local deposition of fibrin forms a meshwork surrounding aggregated platelets to form a stabilised clot that seals the site of vascular injury and prevents blood loss.

In the dynamic process of thrombin generation in blood, some of the thrombin generated during the early stages of coagulation enters a feedback activation loop. This influences the clotting cascade by activating factors V and VIII. Once activated, these factors enhance the function of enzymes involved in the further, more sustained generation of thrombin [Figure 1]. Thrombin activates factors V and VIII in a similar manner by excision of their central B domains. In Factor V, R709, R1018 and R1545 are cleaved leaving an A1-A2 domain fragment, ionically associated with the A3-C1-C2 domains. In factor VIII, the corresponding and analogous cleavages are R372, R740, R1649 and R1689, again leaving the A1-A2 fragment non-covalently associated with the A3-C1-C2 fragment.

Thrombin proteolytically activates factor XIII, a procoagulant reaction that is enhanced by its cofactor fibrin. Once thrombin cleaves the factor XIII A subunit after R37, an amino-terminal activation peptide is released. This exposes the active-site C314 residue to generate the active transglutaminase that stabilises deposited fibrin fibrils by covalent cross-linking [Figure 1].

During normal haemostasis the majority of thrombin generation occurs on the surface of activated platelets that form the primary haemostatic plug. Platelets can be activated by a number of agonists, including thrombin, which specifically activates the platelet by interacting with and cleaving protease activated receptors (PARs) [11]. Cleavage of the PAR1 receptor is at R41, releasing an intramolecular ligand that self associates with one of its extra-cellular loops causing receptor-induced signalling [Figure 1].

A second platelet activation mechanism involves proteolysis of glycoprotein (Gp)V, part of the GpIIb-IX-V complex on circulating platelet surface. This in turn yields hyper-responsive platelets [12]. Thrombin can also proteolytically activate factor XI (another clotting cascade serine protease) under rather defined conditions requiring anions such as dextran sulphate. However, in the presence of activated platelets, a more likely physiological activation process occurs that involves binding of GpIIbα as a cofactor to this reaction, thereafter resulting in cleavage of factor XI after R369 [1].

In addition to the procoagulant activities described above, thrombin also participates in interactions/reactions that elicit anticoagulant function. Thrombin generated adjacent to endothelial cells lining the vessel lumen can bind to its cofactor for this purpose thrombomodulin on the surface of these cells in a high affinity complex [14]. Once bound in the thrombin-thrombomodulin complex, thrombin’s substrate specificity is redirected from procoagulant to anticoagulant reactions. Procoagulant reactions are impeded (in part due to the occupancy of exosite I by thrombomodulin) and activation of protein C is enhanced many fold. This occurs by cleavage of protein C at R169 and the subsequent release of an activation peptide. Activated protein C is an anticoagulant protease that inhibits the function of factors V and VIIIa, and in turn reduces further thrombin generation [Figure 1]. The thrombin-thrombomodulin complex also activates the carboxypeptidase, thrombin-activated fibrinolysis inhibitor (TFPI) by specific proteolysis at R92 [15, 16]. The rate of this activation is ~1000 greater than by thrombin alone. Activated TFPI stabilises fibrin clots through proteolytic removal of terminal lysine residues from fibrin. The clipping of these amino acids disrupts the binding sites for fibrinolytic proteins that destabilise the fibrin meshwork.

Physiologically, the half-life of thrombin in plasma is ~10–15 seconds largely through the inhibitory properties of the plasma SERPIN, antithrombin. Inhibition of thrombin by antithrombin is reversible. However, by first cleaving R393 of the reactive ‘bait’ loop of antithrombin, thrombin becomes locked in an irreversible complex and thereafter is cleared from circulation. This reaction is accelerated by the glycosaminoglycan cofactors heparin and heparan sulphate which approximate the reactants [17]. In a similar process involving cleavage after L444 of another SERPIN, heparin cofactor II, (in the presence of the glycosaminoglycans) thrombin inhibition can be again further augmented [18].

The multiple interactions of thrombin outlined above pose the question of how thrombin activity is specifically coordinated during haemostasis to produce a haemostatic plug only at the site of injury and not over intact endothelium [19]. Occupancy of its surface exosites (I and II) by the above cofactors for many of its reactions [20] provides a mechanism through which its activity can be directed. These exosites interact with cofactors to enhance many of the reactions of thrombin. As cofactors have such a profound influence upon thrombin function, the direction/pathway that thrombin takes becomes more closely controlled by the availability of it cofactors, and therefore the competition of these cofactors for their respective exosites [21].

**Pharmaceutical approach to inhibition of thrombin**

The complexity of the coagulation system allows a number of novel therapeutic strategies for development of anticoagulant drugs. Currently, long term anticoagulation is mostly achieved with oral anticoagulants such as warfarin. For short term treatment, heparin and low MW heparins are used. Warfarin reduces the levels of many clotting factors (including factor VII, factor IX, factor X and prothrombin). The heparins indirectly (by enhancing antithrombin action) inhibit factor Xa and thrombin. A new panel of potential therapeutic agents has been designed to target specific steps in the coagulation system [22]. Two such agents that target thrombin are ximelagatran (a prodrug of melagatran) and
dabigatran (of which the prodrug is dabigatran etexilate). Despite excellent outcomes from clinical trials in terms of anticoagulation which have validated the approach of targeting thrombin for this purpose, ximelagatran caused liver toxicity and is unsuitable for long term therapy. Despite this set back, it must be only a matter of time before a low toxicity and effective direct thrombin inhibitor is available for clinical use.

References

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