Review

An ensemble view of thrombin allostery

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Abstract

Thrombin is the central protease of the coagulation cascade. Its activity is tightly regulated to ensure rapid blood clotting while preventing uncontrolled thrombosis. Thrombin interacts with multiple substrates and cofactors and is critically involved in both pro- and anticoagulant pathways of the coagulation network. Its allosteric regulation, especially by the monovalent cation Na^+, has been the focus of research for more than 30 years. It is believed that thrombin can adopt an anticoagulant ('slow') conformation and, after Na^+ binding, a structurally distinct procoagulant ('fast') state. In the past few years, however, the general view of allostery has evolved from one of rigid structural changes towards thermodynamic ensembles of conformational states. With this background, the view of the allosteric regulation of thrombin has also changed. The static view of the two-state model has been dismissed in favor of a more dynamic view of thrombin allostery. Herein, we review recent data that demonstrate that apo-thrombin is zymogen-like and exists as an ensemble of conformations. Furthermore, we describe how ligand binding to thrombin allosterically stabilizes conformations on the continuum from zymogen to protease.

Keywords: coagulation; NMR spectroscopy; plasticity; protease; zymogen.

Introduction: thrombin function

The coagulation cascade (Figure 1) has evolved to ensure rapid blood clotting after vascular injury and to prevent thrombosis (Davie and Ratnoff, 1964; Macfarlane, 1964). In recent years, the cell-based model of the coagulation response has been established (for reviews see Hoffman, 2003; Monroe and Hoffman, 2006; Hoffman and Monroe, 2007). After vascular injury, tissue factor (TF) is exposed on the surface of subendothelial cells and binds coagulation factor VIIa (fVIIa). Small amounts of fVIIa circulate in the blood, but in a low-activity (zymogen-like) state until it interacts with TF. The fVIIa-TF complex then activates the zymogen precursors fVII, fIX and fX. Active fXa cleaves membrane-bound prothrombin (fII) at two sites and liberates activated thrombin (fIIa) (initiation phase). Thrombin binds to glycoprotein Ibα (GpIbα) expressed on the surface of platelets and cleaves protease activated receptor 1 (PAR-1). This leads to the activation of platelets and a flipping of its membrane leaflets to present a negatively charged phospholipid surface. Thrombin also amplifies its own formation by activating fXI which in turn activates fIX. Factor IXa together with its cofactor fVIIIa, also activated by thrombin, forms the tenase complex to activate more fX. The prothrombinase complex consisting of fVa and fXa forms on negatively charged surfaces to generate a burst of thrombin (amplification and propagation phases). In this phase enough thrombin is generated to efficiently cleave fibrinogen to fibrin. The fibrin monomers polymerize to form the blood clot, which is crosslinked and stabilized by the transglutaminase fXIIIa, another substrate of thrombin. To attenuate the coagulation response, thrombomodulin (TM) binds to thrombin and shifts its activity away from procoagulant substrates towards the anticoagulant protein C (PC). Activated protein C (APC) then cleaves and inactivates fVa and fVIIIa. Active thrombin that has escaped the site of vascular injury can bind to glycosaminoglycans (GAGs) present on the surface of endothelial cells to enhance its inhibition by members of the serpin family, antithrombin (AT) and heparin cofactor II (HCII). The inactivated thrombin-serpin complex is released from GAGs and cleared from the blood by the low-density lipoprotein receptor-related protein (LRP) (attenuation phase). The active role that thrombin plays in each of the phases of coagulation has been referred to as 'Thrombin's Life Cycle' (Huntington, 2008a). Understanding the paradoxical ability of thrombin to be both pro- and anticoagulant and to be at once promiscuous and specific is key to understanding the regulation of coagulation itself. It is a story of cofactors, exosites and allostery.

Thrombin structure

Thrombin is a serine protease with an overall fold similar to the digestive proteases trypsin and chymotrypsin (Figure 2A) (Bode et al., 1989). However, thrombin has some additional structural features that help confer its combination of promiscuity and specificity. Thrombin consists of a 36 residue light chain linked to the catalytic 259 residue heavy chain by a
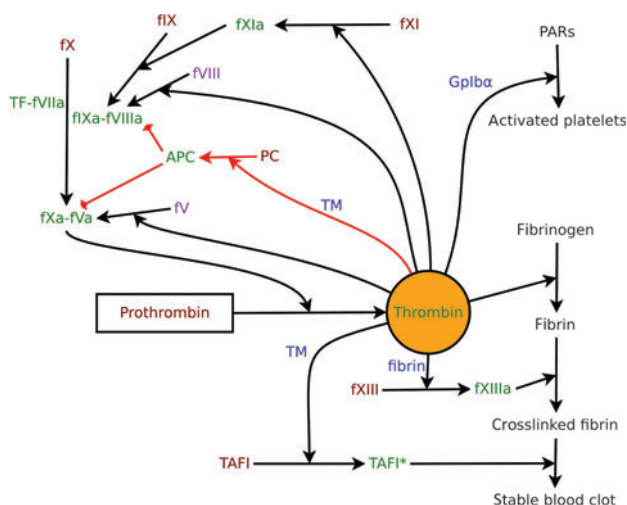
Figure 1 A schematic representation of the blood coagulation cascade.
Thrombin is the central molecule of the coagulation cascade and activates zymogens (maroon-to-green) or procofactors (purple). Some of the reactions are assisted by cofactor binding to thrombin (blue). Thrombin is involved in both procoagulant (black) as well as anticoagulant (red) pathways.

disulfide bond. The protease domain is formed by N-terminal and C-terminal β-barrel domains with the active site consisting of the catalytic triad His57, Asp102 and Ser195 (chymotrypsin template numbering; Bode et al., 1989) between the two barrels (Figure 2A and B). The oxyanion hole formed by the backbone amides of Gly193 and Ser195 stabilizes the transition state during proteolytic cleavage (Figure 2B). Compared to the digestive proteases, the active site of thrombin is inside a relatively deep negatively charged canyon-like cleft formed by the 60s-loop and the γ-loop (or 140s-loop) (Figure 2C). This deep cleft is one of the features that render thrombin specific towards its various macromolecular substrates. Thrombin cleaves its substrates after arginine, the side chain of which is accommodated by the S1 specificity pocket (traditional protease-substrate nomenclature; Schechter and Berger, 1967). This pocket is formed by residues of the 180s-loop and the 220s-loop (Figure 2B). At the bottom of this pocket, Asp189 forms a salt bridge with the positively charged P1 Arg residue of the substrate (Figure 2B).

In addition to the active site, thrombin has two other macromolecular binding sites, anion binding exosites I and II (Figure 2C). Both are positively charged, but exosite I also has a hydrophobic character (Huntington, 2005). Exosite I has important functions during all phases of coagulation; PAR-1 interacts with it in the amplification phase, fibrinogen in the propagation phase and TM at the beginning of the attenuation phase. The C-terminal portion of hirudin, a thrombin inhibitor from the medicinal leech *Hirudo medicinalis*, known as hirugen, also interacts with exosite I (Skrzypczak-Jankun et al., 1991). Exosite II is the more basic of the two sites and is traditionally regarded as an anticoagulant site because it binds heparin or other GAGs to bridge serpins to thrombin (Carter et al., 2005). However, it is also involved in the recognition

of fV and fVIII, and binds to GpIbα to localize thrombin to the platelet surface to stimulate the cleavage of PAR-1 (Huntington, 2005).

The fourth site involved in the regulation of thrombin activity is the Na^+-binding site, formed by the 180s- and 220s-loops. The latter is also known as the Na^+-binding loop because the Na^+ ion is coordinated by the backbone oxygen atoms of Arg221a and Lys224 and by four water molecules (Figure 2B) (Zhang and Tulinsky, 1997). The Na^+-binding site is directly linked to functionally important features of thrombin. The 180s-loop and the Na^+-binding loop form the S1 specificity pocket, and the catalytic serine and oxyanion hole are covalently linked to the Na^+-binding loop via a disulfide bridge between Cys220 and Cys191 (Figure 2B). Thus, it is not surprising that Na^+-binding has an effect on the activity of thrombin. Apo-thrombin has low activity towards certain chromogenic substrates *in vitro*, and has accordingly been named 'slow' thrombin (Wells and Di Cera, 1992). After Na^+-binding however, the activity of thrombin against the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNA) increases by approximately 10-fold, as a result of improvements in both K_m and k_{cat} (Orthner and Kosow, 1980). Thus, Na^+-bound thrombin was termed 'fast' thrombin (Wells and Di Cera, 1992). Fast thrombin has been regarded as a procoagulant form because it has higher activity towards fibrinogen and PAR-1, whereas 'slow' (apo-) thrombin is claimed to be an anticoagulant form because it has near normal activity towards PC activation when bound to TM (Di Cera et al., 1997). The K_d for Na^+ is reported to change with temperature, increasing from 20 mM at 25°C to 110 mM at 37°C (Wells and Di Cera, 1992), and thus both slow and fast forms would be populated *in vivo* (plasma Na^+ concentration is 143 mM). However, a recent study showed a much lower temperature dependence on the affinity of thrombin for Na^+, casting doubt on the physiological relevance of Na^+ modulation of thrombin activity (Kamath et al., 2010). Nevertheless, Na^+ is certainly an important ligand of thrombin, and its binding clearly plays a role in thrombin structure and function.

Activation of prothrombin

Prothrombin, the inactive zymogen precursor of thrombin, is a 72 kDa protein consisting of a Gla domain, two kringle domains and the catalytic domain (Figure 3). Classified by cleavage products, the first two domains together are known as fragment-1, the kringle-2 domain as fragment-2, and all three together are called fragment-1.2 (Figure 3). Thrombin is formed after proteolytic cleavage of prothrombin at two sites by the prothrombinase complex. Prothrombinase first cleaves the Arg320-Ile321 bond (Arg15-Ile16 of thrombin) to form the intermediate meizothrombin, followed by cleavage of the Arg271-Thr272 bond (cleavage at this site alone results in a zymogen form known as prethrombin-2) (Figure 3) (Krishnaswamy et al., 1986). The first cleavage releases the newly formed N-terminus of Ile16 which then folds back into the core of the protease domain (the activation pocket) to make a salt bridge with the side chain of Asp194 (Figure 2B).
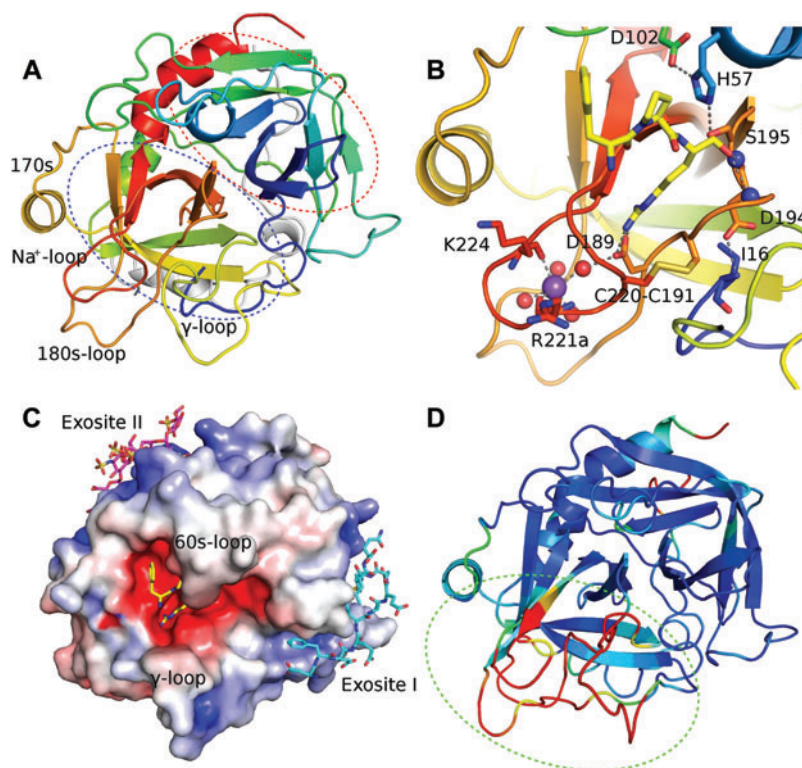
Figure 2 Structural features of thrombin.
(A) Ribbon diagram of thrombin in the standard orientation. The heavy chain is colored from blue to red from the N- to the C-terminus. The light chain is colored gray. Functionally important surface loops are indicated and the N- and C-terminal β-barrels are marked with red and blue ovals, respectively. (B) Detail of the Na⁺-binding site, the S1 pocket, the active site and the activation pocket. The Na⁺ ion (purple sphere) is coordinated by R221a, K224 and four water molecules (red spheres). The active site (His57, Asp102, Ser195) including the oxyanion hole (blue balls) is linked to the Na⁺-loop via the C191-C220 disulfide bond. The active site is shown with the inhibitor PPACK (yellow sticks). The activation pocket with the I16-D194 salt bridge is also indicated. (C) Electrostatic surface of thrombin. The negatively charged active site cleft (shown with PPACK) is formed by the 60s- and γ-loops. The two positively charged anion-binding exosites are shown with their ligands hirugen (cyan sticks) and heparin (magenta sticks). (D) Ribbon diagram of prethrombin-2 colored according to the Cα RMSD between prethrombin-2 (PDB code 1hah) and active thrombin (1hag). The gradient ranges from no differences (blue) to differences larger than 2.7 Å (red). The zymogen activation domain is highlighted by the green oval.

This event stabilizes regions of the 180s-, Na⁺-binding and γ-loops (zymogen activation domain), leading to the correct formation of the S1 pocket and the oxyanion hole (Figure 2D). Although cleavage of the Arg15-Ile16 bond is a crucial step during physiological thrombin formation, it is not absolutely necessary for expression of activity. Formation of a salt bridge between Asp194 and exogenous peptides or proteins such as staphylocoagulase can also 'activate'
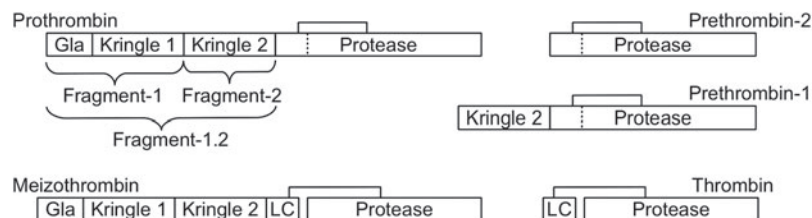

Figure 3 Domain organization of prothrombin and activation products.
Prothrombin consists of a Gla-domain, two kringle domains and the protease domain. Cleavage in the protease domain (Arg320-Ile321 in prothrombin, or Arg15-Ile16 in thrombin using chymotrypsin numbering) leads to formation of meizothrombin, whereas cleavage at Arg271-Thr272 leads to formation of prethrombin-2 and fragment-1.2. Further cleavage of either of the intermediates results in thrombin formation. For *in vitro* analysis prethrombin-1 can be formed, consisting of the kringle 2 domain and the uncleaved protease domain. Fragment-1.2 can be further divided into fragment-1 (Gla-domain and kringle 1) and fragment-2 (kringle 2). Only the disulfide bridge between the light chain (LC) and heavy chain of the protease domain is indicated.

prothrombin via a mechanism termed 'molecular sexuality' (Bode, 1979; Friedrich et al., 2003). Activation through insertion of an N-terminal peptide is universal for all serine proteases, however, there is a high degree of variation in the level of activation. Coagulation factor VIIa is an example of a protease that is cleaved at the 15–16 bond, but does not stably incorporate the N-terminus into the activation pocket (Higashi et al., 1994). Thus, fVIIa is a zymogen-like protease and remains inactive until it binds to its cofactor TF (Higashi et al., 1994; Ruf and Dickinson, 1998). It is also possible for uncleaved zymogens to express protease activity, as exemplified by single-chain tissue plasminogen activator (tPA). Indeed, activation cleavage only increases tPA activity by 5- to 10-fold, whereas activation of trypsinogen to trypsin increases activity by seven orders of magnitude (Renatus et al., 1997).

Thus, although the activation of zymogens is generally driven by the catalytic cleavage event at the 15–16 bond, it is possible for zymogens to be activated by peptide or protein binding, for cleaved proteases to maintain a zymogen-like state, and for zymogens to express full proteolytic activity. All these examples show that the transition from zymogen to protease is not a simple two-state transition governed by cleavage, but a more complex continuum of states along a trajectory from zymogen to protease (Kamath et al., 2010).

Why prothrombin is inactive

The first crystal structures of zymogens, chymotrypsinogen and trypsinogen, surprisingly revealed that the catalytic triad was fully formed in a functional conformation (Freer et al., 1970; Fehlhammer et al., 1977). The main structural differences between chymotrypsinogen and chymotrypsin were that the loops of the activation domain were in different conformations, resulting in a malformed S1 specificity pocket and oxyanion hole for chymotrypsinogen (Freer et al., 1970). Similar features were observed in trypsinogen, however, the loops of the activation domain were not visible in the electron density map, indicating a high degree of flexibility (Fehlhammer et al., 1977). The same loops are also not visible in a recently released crystal structure of prethrombin-2 bound to fragment-2 (3k65). Prethrombin-2 is the simplest zymogen form of thrombin, consisting only of the uncleaved protease domain and thus is comparable to the zymogens of the digestive enzymes. For prothrombin and prethrombin-2, it has been shown by H/D exchange experiments that the C-terminal β-barrel including the zymogen activation domain is more flexible in the zymogen than in the active protease (Koeppe and Komives, 2006).

Thus, the structural differences between the zymogen and protease states of trypsinogen/trypsin, chymotrypsinogen/chymotrypsin and prethrombin-2/thrombin are conserved. In the zymogen forms of thrombin, however, not only the active site has to be non-functional, but to prevent premature interaction with cofactors and substrates, both exosites also have to be out of commission. Exosite I is accessible in prothrombin but is in a different conformational state than in active

thrombin (Liu et al., 1991). Compared to active thrombin, prothrombin has an approximately 100-fold weaker affinity for hirugen, whereas prethrombin-1 and prethrombin-2 only have 10- to 20-fold weaker affinity (Anderson et al., 2000, 2003). Recent experiments showed that addition of fragment-1.2 has a profound effect on prethrombin-2 and seems to lead to conformational changes in exosite I resulting in a reduction in affinity for hirugen (Anderson and Bock, 2003). Exosite II on the other hand is fully formed in prethrombin-2, but binds with high affinity to prothrombin fragment-2 (Anderson et al., 2003), blocking its interactions with other partners (Arni et al., 1993). Thus, in addition to the active site, both critical anion binding exosites are inoperative in prothrombin.

The fourth binding site relevant to thrombin activity is the Na$^+$-binding site, which is formed by portions of the zymogen activation domain (Orthner and Kosow, 1980; Wells and Di Cera, 1992; Zhang and Tulinsky, 1997). The binding of Na$^+$ is allosterically linked to exosite I in thrombin and meizothrombin, whereas prothrombin, prethrombin-1 and prethrombin-2 show no allosteric linkage (Kroh et al., 2007). Together with the observation that the Na$^+$-binding site is not properly formed in prethrombin-2, this suggests that the zymogen forms of thrombin cannot bind Na$^+$ (Vijayalakshmi et al., 1994; Kroh et al., 2007). The fact that both meizothrombin and thrombin are able to bind Na$^+$ indicates that formation of the Ile16-Asp194 salt bridge is necessary for Na$^+$ binding and may be linked events.

Thus, the inactivity of prothrombin is the result of enhanced flexibility or conformational fluctuations resulting in the improper/incomplete formation of the Na$^+$-binding site, the S1 specificity pocket, the oxyanion hole and exosite I.

Thrombin allostery

Traditionally, allostery is seen as a binding event at one site (allosteric site) that induces a distinct conformational change at another site (e.g., active site) (Monod et al., 1965; Koshland et al., 1966). This model states that there are two different discrete conformations: the ligand-free low activity state and the ligand-bound high activity state (Figure 4A) (Tsai et al., 2009). A similar allostery model was initially proposed for the activation of thrombin by Na$^+$. Na$^+$-free ('slow') thrombin shows low activity, whereas Na$^+$-bound ('fast') thrombin has higher activity (Wells and Di Cera, 1992; Ayala and Di Cera, 1994). Later, this scheme was extended to a three-state model (Figure 4B) (Bah et al., 2006; Gianni et al., 2007). Ultra-rapid kinetics experiments showed that thrombin can exist in an inactive E* state which is incapable of binding Na$^+$ or substrates. The conversion to the low activity E state enables thrombin to bind Na$^+$ to form the Na$^+$-bound E:Na$^+$ state (Bah et al., 2006; Gianni et al., 2007). In the previous nomenclature, the low activity E state would correspond to 'slow' thrombin and the high activity E:Na$^+$ state would be 'fast'. It was estimated that at physiological Na$^+$ concentrations, approximately 60% of the thrombin generated would exit in the 'fast' form, 40% in the 'slow' form and <1% in the
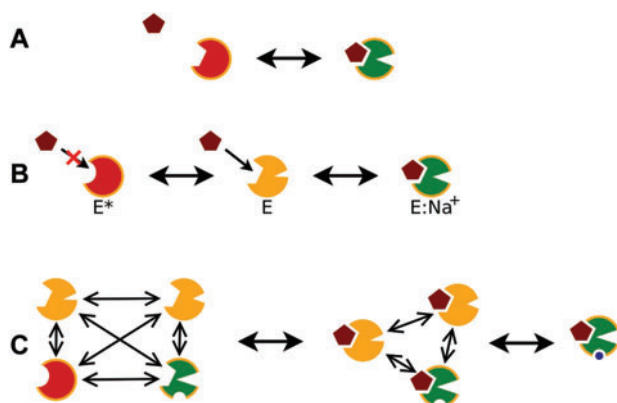
Figure 4 Models of protein allostery.
(A) The classic model of allostery. Binding of a ligand (brown) to the allosteric binding site of an inactive (red) protein induces a structural change at a different site (e.g., active site) and thus induces protein activity (green, open 'mouth'). (B) The three state allosteric model of thrombin. The inactive (red) E* form of thrombin is incapable of binding Na$^+$. A slow transformation to the E form (orange, half-open 'mouth', low activity) enables Na$^+$ binding, and Na$^+$ binding to E results in the fully active E:Na$^+$ ('fast') form (green, fully opened 'mouth') of thrombin. (C) Ensemble view of allostery. Without any ligands, the protein exists as an ensemble of different conformations that may even include the fully active conformation. Ligand binding to allosteric sites shifts the equilibrium towards more active states and may increase affinity for other allosteric modulators, which then further shifts the equilibrium towards the most active state.

E* form, raising questions of the physiological relevance of the latter (Wells and Di Cera, 1992; Bah et al., 2006).

In addition to the allosteric linkage between the Na$^+$-binding site and the active site, a similar allosteric linkage has been shown between exosite I and the active site. Hirugen binding to exosite I affects the hydrolysis of chromogenic substrates by thrombin (Liu et al., 1991; Ayala and Di Cera, 1994). For S-2238, for example, a 30% increase in the rate of hydrolysis was seen after hirugen binding (Liu et al., 1991). This effect is more profound for 'slow' thrombin, indicating that hirugen and Na$^+$ can induce similar allosteric changes (Ayala and Di Cera, 1994). Experiments also suggest that exosite I ligation and active site binding induce similar structural changes (Parry et al., 1993). Thrombomodulin has the unique ability of moving thrombin specificity towards the anticoagulant substrates and much effort has been made to determine the allosteric changes induced by TM. It was shown that TM binding increases the reactivity with inhibitors such as BPTI and PCI (Rezaie et al., 1995, 1998; De Cristofaro and Landolfi, 1999), and it also increases the activity of thrombin towards PC by more than 1000-fold (Xu et al., 2005). A positive effect of hirugen binding on Na$^+$ binding has also been observed (Ayala and Di Cera, 1994). The converse also holds, with TM and hirugen binding more tightly to the 'fast' form of thrombin than to the 'slow' form, indicating an allosteric linkage between the Na$^+$-binding site and exosite I (Vindigni et al., 1997).

The 'ensemble' view of protein allostery

In recent years, the classic model of allostery has been extended. It is now believed that instead of inducing a conformational change from a rigid inactive state to a rigid active state, binding of a ligand to the allosteric site alters the equilibrium position of an ensemble of conformations towards an active state (Figure 4C) (Tsai et al., 2008, 2009; Boehr et al., 2009; Hilser, 2010). Thus, allostery is a thermodynamic phenomenon which is driven by changes in the entropy and/or enthalpy (Tsai et al., 2008). In the following sections we will discuss how the emerging change in the definition of allostery affects our understanding of allostery in thrombin. Many insights into thrombin allostery were derived from fluorescence methods and protein crystallography. These methods led to a static view of thrombin allostery, with a concerted conformational change from a 'slow' form to a 'fast' form upon ligand binding. This view, however, is beginning to change. Research done by our group and others looking into the behavior of thrombin in solution using hydrogen/deuterium exchange, mutagenesis studies, isothermal titration calorimetry (ITC) and, most recently, NMR spectroscopy has led to a more dynamic view of thrombin allostery.

Apo-thrombin is zymogen-like

As mentioned earlier, the zymogen-to-protease transition is driven by cleavage of the 15–16 bond, but is not as concerted as once believed. This is exemplified by the zymogen-like protease fVIIa, the proteolytically active zymogen tPA, and activation of zymogens by ligands, including peptides and staphylocoagulase. In recent years, this paradigm has also changed for thrombin through work done by our group and others. De Filippis and coworkers used spectroscopic methods to determine the structural effects of Na$^+$ on thrombin (De Filippis et al., 2002, 2005). They concluded that slow thrombin has high structural heterogeneity and that Na$^+$ has stabilizing effects in the active site cleft and surface loops. We have employed NMR spectroscopy to determine the structural and dynamic effects of thrombin ligation. By determining backbone resonance assignments for seven different ligation states, from ligand-free apo-thrombin to fully liganded thrombin (PPACK-inhibited, with Na$^+$ and hirugen bound), we were able to obtain residue-specific (high resolution) information (Lechtenberg et al., 2010) (Figure 5). We used the fact that signals in NMR spectroscopy are characterized by a structural component (chemical shift) and a dynamic component (line shape). Apart from the uniform contribution from the overall tumbling of a given protein, the line-width of a resonance signal is broadened by chemical or conformational exchange processes between various states on the microsecond-to-millisecond timescale. In some cases, exchange broadening leads to a loss of resonance peaks due to attenuation of signals to the noise level. Protein conformational changes generally take place in this timescale (Boehr et al., 2006).

The ^{1}H-^{15}N-TROSY NMR spectra thus provide a finger print of all non-proline backbone residues. For apo-thrombin,
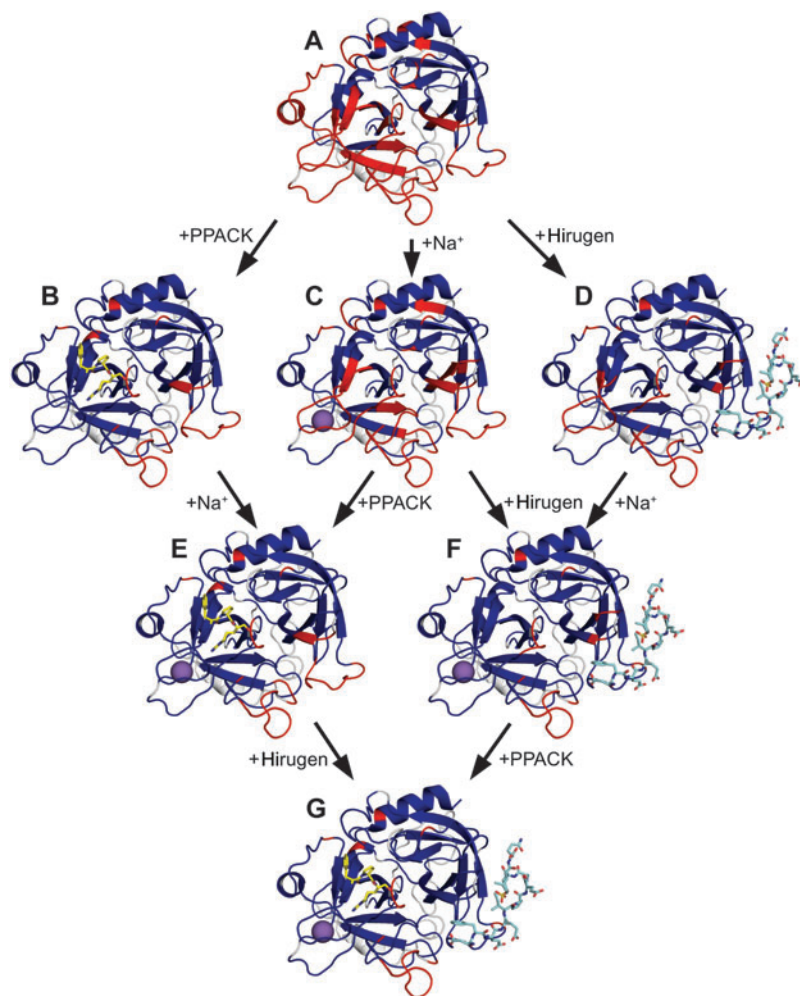
Figure 5 Apo-thrombin is a zymogen-like, flexible molecule that is stabilized by ligand binding.
Cartoon representations of the protease domain of thrombin are colored according to NMR backbone resonance assignment status (assigned residues are in blue; residues which could not be assigned due to line broadening are colored red; proline residues and the light chain are in gray). Apo-thrombin is in a flexible state, which most likely consists of an ensemble of conformations. Thrombin is stabilized by binding to PPACK (yellow sticks), hirugen (cyan sticks) and Na$^+$ (purple sphere).

only 50% of the residues gave rise to a resonance peak that could be assigned (Figure 5A). Most of the N-terminal β-barrel was assigned in the apo-form, but the C-terminal β-barrel, including functionally important regions, could not be assigned, due to chemical exchange broadening. These included the Na$^+$-binding loop, the 180s-loop and the active site loop with the oxyanion hole (Figure 5A). No direct signals for the N-terminal residue Ile16 can be observed by this method because of the lack of a peptide bond; however, the fact that resonances for the next eight residues were not observed suggests that the N-terminus was not stably incorporated into the activation pocket (Figure 5A). This would explain the relatively low activity of apo-thrombin against the chromogenic substrate S-2238 and the higher fibrinogen clotting time (Orthner and Kosow, 1980). Lack of stable N-terminus insertion has also been demonstrated for the zymogen-like protease fVIIa, for which it was shown that the N-terminus of the apo-enzyme is more readily available

for chemical modification than the TF-bound form (Higashi et al., 1994). The TROSY spectrum of apo-thrombin indicates that it is a dynamic molecule characterized by the interconversion between multiple conformers, particularly in regions associated with the zymogen-to-protease conversion.

Ligands shuttle thrombin towards its active state

Apo-thrombin is conformationally dynamic in solution and shows poor activity. Upon ligand binding, however, conformational flexibility is reduced and activity is increased. This was shown in our NMR experiments, where stepwise ligation led to lower conformational variations as determined by more signals being present in the NMR spectra. PPACK binding to the active site had the strongest effect. It stabilized the whole zymogen activation domain and only the exosite I region and

the γ-loop were not in a stable conformation (Figure 5B). The binding of hirugen or Na$^+$ to apo-thrombin had similar, although weaker effects. Hirugen binding stabilized exosite I, but also allosterically stabilized large parts of the zymogen activation domain, including the 180s-loop, which is part of the S1 pocket, and also other parts of the C-terminal β-barrel (Figure 5D). Some functionally important regions such as the Na$^+$-binding loop and the very N-terminal residues of the protease domain, however, remained dynamic. Na$^+$ binding also stabilized parts of the zymogen activation domain, including the 180s-loop and parts of the C-terminal β-barrel (Figure 5C). Interestingly, the Na$^+$-binding loop itself was not stabilized, probably due to the fast exchange rates of the Na$^+$ ion (Bah et al., 2006; Gianni et al., 2007). The binding of an additional ligand generally led to further rigidification of thrombin. The one exception is the addition of Na$^+$ to PPACK-bound thrombin, which only induced minor local changes to the Na$^+$-binding residues (Figure 5B and E). The combined binding of hirugen and Na$^+$ results in stabilization of the complete zymogen activation domain and the N-terminal residues (Figure 5F). Hirugen uniquely stabilizes the exosite I loops and the stems of the γ-loop (Figure 5D, F and G). Thus, PPACK alone can lock thrombin in the active state and prevent further conformational change, while thrombin otherwise requires a combination of exosite I and Na$^+$ binding to achieve full stabilization of the active state.

Thrombin exists as a conformational ensemble

Conformational stabilization of thrombin via ligation has also been described by other groups. H/D exchange experiments showed decreased exchange in exosite I and the γ-loop upon PPACK binding (Croy et al., 2004). Treuheit et al. (2011) showed that binding of ligands to exosite I (TM or an aptamer) resulted in reduced dynamics of thrombin and therefore a lower entropic cost of binding of a second ligand to the active site. A continuum of states from zymogen-like to protease-like was demonstrated by Kamath et al. (2010) using fragment-1.2 as a probe for 'zymogenicity' of thrombin mutants and ligation states (Kamath et al., 2010). It is thus possible to discuss the relationship between thrombin structure, dynamics and activity in terms of an 'ensemble view of allostery' (Hilser, 2010) or of 'conformational selection' (Boehr et al., 2009). A well-characterized example is provided by work conducted on the adenylate kinase from *Aquifex aeolicus* (Henzler-Wildman et al., 2007). In that report, biophysical (X-ray crystallography, NMR spectroscopy, FRET) and computational (molecular dynamics simulations) methods were used to determine the conformational transition between the inactive 'open' form and the catalytically active 'closed' form. These experiments showed that the inactive state consists of an ensemble of conformations that fluctuate in a microsecond-to-millisecond timescale (Henzler-Wildman et al., 2007). The same could be said for thrombin. The differences between crystal structures of putative 'slow' and 'fast' forms of thrombin and the regions involved in the Na$^+$-driven activation of thrombin have been analyzed in detail in the past

(Huntington, 2008b). New insights from our NMR studies and experiments published by other groups also suggest that apo-thrombin (or 'slow' thrombin) is not a static structure, but is indeed remarkably dynamic and likely to exist in several conformations in solution. This would explain why crystal structures of putative 'slow' thrombins show a large conformational variation and include structures that are predictably completely inactive and others that are indistinguishable from 'fast' thrombin (Huntington, 2008b). An overlay of 15 crystal structures of 'slow' thrombins confirms this ensemble view of allostery (Figure 6A). The same regions that show enhanced flexibility in the NMR experiments also sample various conformations in the crystal structures. These regions include the zymogen activation domain, the 180s-loop, the Na$^+$-binding loop, the γ-loop, the 170s-loop/helix and the active site region. Additional to these regions, the exosite I loops, and the 60s- and 90s-loops show enhanced flexibility (Figure 6A). Consistent with the NMR results, the N-terminal β-barrel seems to be more stably folded than the C-terminal barrel. Comparison of several structures of 'fast' thrombin shows that these structures exhibit much less conformational variation (Figure 6B). Especially the zymogen activation domain, which seems to be in a well-defined state, and also the stems of the γ-loop and the exosite I loops show less flexibility. Regions still exhibiting conformational variability in the 'fast' state include the 60s-loop, the 90s-loop and the γ-loop. The latter was also shown to be dynamic even in the fully liganded form of thrombin in the NMR experiments (Lechtenberg et al., 2010). Taken together, the new data suggest an ensemble view of thrombin allostery (Figure 4C). In this model, apo-thrombin exists in an ensemble of conformations that includes inactive as well as low-activity and high-activity conformations. Binding of one ligand changes the equilibrium within the ensemble and may enrich conformations with higher activity and higher affinity for a second ligand (Figure 4C). After binding of the second ligand, thrombin is fully stabilized in the active conformation and expresses full activity (Figure 4C).

Conclusion

It is well-established that thrombin is an allosterically regulated enzyme. Research has focused on the allosteric effects of the monovalent cation Na$^+$ which has been shown to modulate thrombin activity (Orthner and Kosow, 1980; Wells and Di Cera, 1992). Owing to the unique effects of Na$^+$ on the activity of thrombin, the terms 'slow' and 'fast' were utilized for apo-thrombin and Na$^+$-bound thrombin, respectively (Wells and Di Cera, 1992), and were assumed to correspond to distinct conformational and functional (anticoagulant/procoagulant) states. However, despite the determination of crystal structures of several putative 'slow' forms of thrombin, there is still no consensus of what the structural features of 'slow' thrombin are. Indeed, recent experiments suggest that discrimination between 'slow' and 'fast' thrombin is somewhat artificial and is probably promoted by the initial definition of allostery as a transition between two distinct, rigid structures. Recent experiments suggest a more dynamic view, where
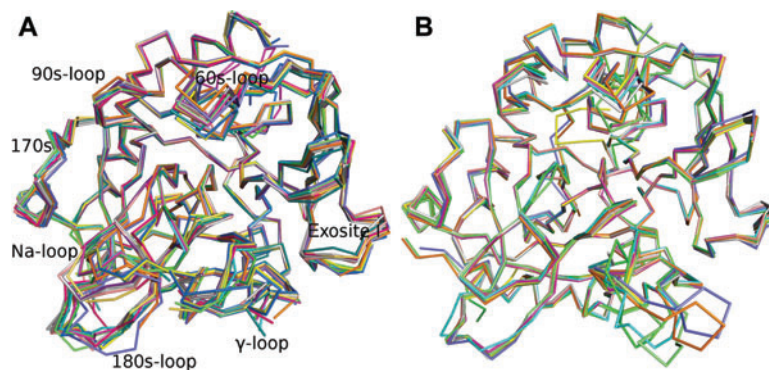
Figure 6 Ensemble view of thrombin crystal structures.
(A) Overlay of 15 'slow' thrombin crystal structures [1mh0 (2 copies), 1rd3 (2 copies), 1sgi (2 copies), 1tq0 (2 copies), 2afq (2 copies), 2gp9, 3bei, 3ee0, 3jz2, 3s7h] in ribbon representation. The 'slow' structures show large variations in the 180s-loop, Na⁺-binding loop, active site loop, and the γ-loop. Additional flexible regions include the two exosite I loops, the 60s- and 90s-loops and the 170s-loop/helix. (B) Overlay of nine 'fast' thrombin structures [1hah, 1hxf, 1jou (3 copies), 1ppb, 1sg8 (2 mol), 1tmu] in ribbon representation. The conformational similarities between the 'fast' structures are much greater than in the 'slow' structures, especially in the 180s-loop, Na⁺-binding loop, active site loop, stems of the γ-loop and the 170s-loop/helix. The two exosite I loops are also less variable but still show some flexibility. As confirmed by the NMR experiments, the γ-loop appears flexible even in the fully liganded thrombin forms.

apo-thrombin is present in an ensemble of conformations in solution with characteristics similar to the zymogen. Ligand binding captures the more active states and effectively pulls the equilibrium to the right (increased activity). This view of thrombin allostery explains the difficulties in determining the structural features of 'slow' thrombin. Instead of the one 'slow' structure of thrombin there exists an ensemble of 'slow' thrombins with features ranging from zymogen-like to protease-like in solution, probably represented by the conformational differences in all crystal structures of putative 'slow' thrombins.

The apparent plasticity of apo-thrombin might also explain the paradox between specificity and promiscuity, because diverse ligands and substrates could induce their own binding conformations in exosite I and the active site. But will thrombin ever exist in the apo-state *in vivo*? Previous studies suggested a ratio of approximately 60:40 for Na⁺-bound and Na⁺-free thrombin at the plasma Na⁺ concentration of 143 mM (Wells and Di Cera, 1992; Bah et al., 2006). However, the dissociation constant of 110 mM at 37°C was determined at pH 8. A recent report using ITC at pH 7.4 showed very little temperature dependence on Na⁺ binding to thrombin (Kamath et al., 2010). Indeed, if the reported K_d of 34 mM is correct, then over 80% of thrombin will be in the 'fast' form and, therefore, the apo-state could be considered an *in vitro* artifact. Furthermore, the classification of 'slow' thrombin as an anticoagulant and 'fast' thrombin as a procoagulant is problematic because it was shown long ago that Na⁺ not only increases thrombin activity towards procoagulant substrates but also increases affinity for TM and activity towards the anticoagulant substrate, PC (De Cristofaro et al., 1996). Therefore, the slow-fast, anticoagulant-procoagulant distinctions for apo- and Na⁺-bound forms of thrombin are of dubious physiological relevance. However, our NMR data suggest that even in the Na⁺-bound state, thrombin is sufficiently conformationally labile to allow the induced-fit binding of its many diverse substrates, cofactors and inhibitors during its life cycle.

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