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How Na⁺ Activates Thrombin – A Review of the Functional and Structural Data

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Abstract

Thrombin is often referred to as the *ultimate* blood coagulation protease. This is true in both senses: it is the final protease generated in the series of proteolytic events known as the blood coagulation cascade, and it is the effector of clot formation, cleaving over twelve different substrates and interacting with at least six cofactors. Regulation of thrombin activity is thus of great relevance to determining the correct haemostatic balance, with dysregulation leading to bleeding or thrombosis. One of the most enigmatic and controversial regulators of thrombin activity is the monovalent cation Na⁺. When bound to Na⁺, thrombin adopts a ‘fast’ conformation which cleaves all procoagulant substrates more rapidly, and when free of Na⁺, thrombin reverts to a ‘slow’ state which preferentially activates the protein C anticoagulant pathway. Thus, Na⁺ binding allosterically modulates the activity of thrombin and helps determine the haemostatic balance. Over the last 30 years there has been a great deal of research into the structural basis of thrombin allostery. Biochemical and mutagenesis studies established which regions and residues are involved in the slow→fast conformational change, and recently several crystal structures of the putative slow form have been solved. In this article I review the biochemical and crystallographic data to see if we are any closer to understanding the conformational basis of the Na⁺ activation of thrombin.

Keywords

allostery; cofactor; crystal; recognition; sodium; substrate

Thrombin in haemostasis

Haemostasis (blood clotting) is initiated by damage to the blood vessel wall, exposing the subendothelial tissue to the blood (Figure 1, and for recent reviews see (Davie and Kulman, 2006; Lane *et al.*, 2005; Monroe and Hoffman, 2006; Jesty and Beltrami, 2005)). The subendothelial surface is rich in collagen, which binds to and localises platelets, and in tissue factor (TF), which initiates the protease activation cascade by binding to factor VIIa. Factor VIIa circulates in a zymogen-like, poorly active state, and is allosterically activated through its interaction with TF. The TF-VIIa complex generates factor Xa, which assembles with factor Va on membranes to form the prothrombinase complex. This early prothrombinase converts a small amount of prothrombin to thrombin to initiate clot deposition. At this stage, thrombin cleaves protease activated receptor 1 (PAR1) thereby stimulating platelets to release their granules and flip their membranes so that the outer surface becomes capable of supporting the assembly of the tenase and prothrombinase complexes. Thrombin then activates factor XI which converts IX to IXa; activates factor VIII which assembles with IXa to form the tenase

complex; and activates factor V which is a critical cofactor for the prothrombinase complex. All of the thrombin activities mentioned so far deposit a small primary haemostatic plug and set the stage for rapid and massive thrombin generation.

The growing clot is stabilised in the next stage by the action of thrombin: thrombin converts fibrinogen to fibrin, which spontaneously polymerises; it stabilises the fibrin mesh by activating factor XIII which laterally crosslinks the polymers, and by inhibiting fibrinolysis by activating thrombin activatable fibrinolysis inhibitor (TAFI). When thrombin has fulfilled its procoagulant functions it is either inhibited by circulating serpins, such as antithrombin (AT) and heparin cofactor II (HCII), or it interacts with thrombomodulin (TM) on the surface of the adjacent intact endothelium. By binding to TM, the activity of thrombin is effectively switched from cleavage of prothrombotic substrates to exclusive cleavage of protein C (and TAFI). Activated protein C (APC) then shuts down thrombin generation by inactivating cofactors Va and VIIIa through proteolytic action. Thrombin thus plays critical roles in boosting and attenuating its own formation, and can be thought of as a Janus-faced pro- and anti-coagulant protease (Bode, 2006).

The discovery of the Na⁺ effect

Cofactors such as TM play important roles in determining thrombin substrate specificity (Adams and Huntington, 2006). Another factor that regulates the activity of thrombin is the monovalent cation Na⁺. The effect of Na⁺ was first described in the seminal paper by Orthner and Kosow in 1980 (Orthner and Kosow, 1980). They showed that Na⁺ (and also K⁺ and Rb⁺, but not NH₄⁺, Li⁺ or Ca²⁺) increased the rate of cleavage (k_{cat}/K_m) of several chromogenic substrates in a manner which depended on the substrate used. For 'good' substrates (i.e. low K_m) the K_m was improved only slightly, with most of the increase in rate provided by an increase in k_{cat} , while for 'bad' substrates (high K_m), improved cleavage was primarily caused by lowering the K_m . For everyone's favourite thrombin substrate, S-2238, K_m and k_{cat} were both improved by about 3-fold for an overall enhancement of ~10-fold. This apparent activation by Na⁺ could of course have significance in thrombin cleavage of physiological substrates, such as fibrinogen. Accordingly, Orthner and Kosow showed a Na⁺-dependent decrease in fibrinogen clotting time (when ionic strength was accounted for). The improved recognition and cleavage of substrates was found to be coincident with an apparent change in conformation, as evidenced by changes in UV absorbance difference spectra which reported an alteration in the environment of tyrosine residues. The affinity of thrombin for Na⁺ was studied by both kinetic and spectroscopic techniques, yielding a binding constant in the range of 20mM at 30°C (when excluding the values obtained with 'bad' substrates). Orthner and Kosow thus demonstrated that the specific binding of Na⁺ to thrombin causes a conformational change which results in improved recognition of substrates and inhibitors. In the 28 years since their report, research groups have continued to characterize the effect of Na⁺ binding on thrombin activity by biochemical and structural techniques. However, instead of converging on a common understanding of the mechanism of Na⁺ binding and the functional and structural consequences for thrombin, the field has become mired in differing opinions, contradictions and confused analysis. The goal of this article is to review the published data and deposited crystal structures to see if, with a fresh and objective analysis, a coherent story emerges.

An introduction to thrombin structure

In order to address how Na⁺ binding might alter the functional properties of thrombin, it is first necessary to introduce the well characterised thrombin structure. Thrombin is a typical serine protease of the chymotrypsin family (Bode *et al.*, 1989), composed of two β -barrel domains that join to form the active site cleft (Figure 2A). The mechanism of peptide bond hydrolysis by serine proteases has been worked out in great detail, and involves the precise alignment of

the scissile bond of the substrate (the so-called P1-P1' bond after the nomenclature of Schechter and Berger (Schechter and Berger, 1967), where substrate residues are numbered sequentially to either side) with the catalytic residues of the protease (Figure 2B). Proteolysis occurs through nucleophilic attack by the side chain γ oxygen of Ser195 (chymotrypsin template numbering is used throughout) on the main chain carbonyl carbon of the P1 residue of the substrate. This reaction requires the potentiation of Ser195 O γ by the catalytic diad of Asp102 and His57. The resulting tetrahedral transition state is stabilized by the so-called oxyanion hole, formed by the main chain amides of Gly193 and Ser195, and eventually collapses to the acyl enzyme intermediate. After diffusion of the P' residues out of the active site cleft, a molecule of water is potentiated by the catalytic diad for attack of the acyl enzyme to release the P end and regenerate the active protease. One of the most important factors determining the rate of hydrolysis of a substrate, and thus specificity, is the complementarity of the substrate peptide sequence with the properties of the active site cleft of the protease.

Thrombin has a particularly deep active site cleft which is occasionally referred to as a 'canyon' (Figure 2C). This is due to insertion loops above (the 60-loop) and below (the γ -loop, also known as the autolysis loop) the cleft. The principal subsite interaction is the burying of the consensus P1 Arg side chain into the deep S1 pocket, where it makes hydrophobic interactions down to the guanidine group which forms a two-pronged salt bridge with Asp189 at the base of the pocket. Also critical is the interaction of a hydrophobic, often aromatic P4 residue of a substrate with the aryl binding pocket (S4) of thrombin. The insertion loops can interact directly with the substrate peptide, with the 60-loop helping form the S2 and S4 sites, and the γ -loop potentially forming contacts on the P' side. The loops also contribute to thrombin specificity by restricting access to the active site cleft. Macromolecular protein substrates require long substrate loops to access the deep pocket, or must make direct 'exosite' contacts with the insertion loops.

Thrombin has three other sites relevant to its activity: two anion binding exosites and the Na⁺ binding site (for a recent review see (Huntington, 2005)). Anion binding exosite I (also known as the fibrinogen recognition exosite) is quite hydrophobic in nature and interacts with several important cofactors and substrates, notably fibrin(ogen), TM and PAR1. Exosite II is the more basic of the two sites and was identified by mutagenesis and structural studies as the heparin binding site. It is also the principal site of interaction of the platelet glycoprotein receptor GpIb α . Importantly, all natural thrombin substrates interact with at least one exosite (usually both), either directly or through cofactor mediation. Another site of potential importance, and the focus of this review, is the Na⁺ binding site. The rough position of Na⁺ bound to thrombin was identified in 1995 by Di Cera and colleagues (Di Cera *et al.*, 1995), but the correct coordination was first reported by Zhang and Tulinsky two years later (Zhang and Tulinsky, 1997). Na⁺ was subsequently found in many previously solved thrombin structures by searching for water molecules with the signature octahedral coordination geometry at the identified site. Na⁺ is coordinated to the main chain oxygens of Arg221a and Lys224 and four conserved water molecules (Figure 2D).

The effect of Na⁺ on the physiological activity of thrombin

There are many physiological targets of thrombin, and only a few of them are mentioned in the previous sections. Orthner and Kosow showed that fibrinogen clotting time was decreased in the presence of Na⁺ relative to other salts, indicating that the rate of fibrinogen cleavage was enhanced (Orthner and Kosow, 1980). Subsequent studies showed that both fibrinopeptide A and B are released faster in the presence of Na⁺ (7 and 4-fold, respectively (Dang *et al.*, 1995), or 23-fold for both (Dang *et al.*, 1997), depending on how the data were analysed). Na⁺ acceleration has also been shown for cleavage of PARs (Bush *et al.*, 2006; Di Cera *et al.*, 1997). Surprisingly, direct Na⁺ effects have not been measured for cleavage of other

important substrates, such as factors V, VIII, XI and XIII, and effects must be inferred from Na⁺-binding deficient variants of thrombin (Myles *et al.*, 2001; Myles *et al.*, 2002; Yun *et al.*, 2003; Philippou *et al.*, 2003). In contrast to the procoagulant substrates of thrombin, activation of protein C is been reported to be slightly faster in the absence of Na⁺ (~7-fold in the absence of TM, and 1.25 to 4-fold with TM) (Dang *et al.*, 1995), although another report showed a ~10-fold activation of protein C cleavage upon Na⁺ binding (De Cristofaro *et al.*, 1996). Since Na⁺-bound thrombin generally cleaved substrates at faster rates it became known as 'fast' thrombin, and since the Na⁺-free form was generally less efficient it became known as 'slow' thrombin. Furthermore, it was argued that the slow form was essentially anticoagulant since it cleaved protein C with improved specificity over other substrates (Dang *et al.*, 1995). Thus, thrombin appears to exist in a procoagulant *fast* state when Na⁺ is coordinated and an anticoagulant *slow* state when Na⁺-free.

The relevance of the two thrombin forms in regulating blood coagulation remains unclear, but the apparent temperature dependence of the K_d of thrombin for Na⁺ suggests that the slow and fast forms are equally populated in blood, where the Na⁺ concentration is 143mM (Wells and Di Cera, 1992; Prasad *et al.*, 2003). Although the Na⁺ levels in blood are highly regulated, there are reported cases of thrombosis and bleeding associated with elevated and reduced levels, respectively (Di Cera, 2007). In addition, several mutations adversely affecting Na⁺ binding have been associated in the literature with a bleeding phenotype (Di Cera, 2007). The anticoagulant activity of Na⁺-free thrombin has also been exploited by creating recombinant slow variants that are effective anticoagulants in animal models (Gibbs *et al.*, 1995; Gruber *et al.*, 2002). The data summarised in this section comprise, in a nutshell, the argument that the Na⁺ activation of thrombin is of physiological relevance.

Evidence for global conformational change

Early evidence that Na⁺ induces a large scale conformational change in thrombin was provided by spectroscopic studies. Initial UV difference spectra showed a likely change in the local environment of aromatic residues, particularly tyrosines (Orthner and Kosow, 1980; Villanueva and Perret, 1983). Subsequently, circular dichroism (CD) revealed a significant loss of ellipticity in the far UV region which was interpreted as a loss of secondary structure caused by Na⁺ coordination (Villanueva and Perret, 1983; Ayala and Di Cera, 1994). Near UV CD spectra showed differences which were consistent with a change in the local asymmetry of aromatic residues. Evidence that tryptophan residues were involved was provided by a ~20% increase in the intrinsic fluorescence of thrombin in response to Na⁺ binding (Wells and Di Cera, 1992). A recent report recapitulated the spectroscopic observations and reinterpreted the far UV CD data as indicative of a change in packing of certain aromatic clusters, not a decrease in secondary structure (De Filippis *et al.*, 2005). Indeed, the CD spectra in the absence and presence of Na⁺ significantly overestimate the amount of secondary structure in thrombin, indicating that the packing of disulphide bonds and aromatic side chains contribute significantly to the observed signal. It is therefore likely that the Na⁺-induced conformational change involves loop regions and aromatic side chains (including disulphides), but not the overall secondary structure content or gross tertiary structure of thrombin.

Which regions are involved?

The original observation that the K_m of chromogenic substrates was affected by Na⁺ binding suggested a change in the conformation of the active site cleft. Since the K_m was improved for all substrates tested, it was concluded that Na⁺ binding induced a general opening of the active site cleft, not a specific change in subsite specificity (Orthner and Kosow, 1980). This has been echoed in a multitude of published reports, showing lower K_m 's for substrates and lower K_i 's for active site-directed inhibitors. In the seminal paper by Wells and Di Cera (Wells and Di

Cera, 1992), where the 'slow/fast' nomenclature was first introduced, careful kinetic studies were conducted at a range of viscosities to determine individual rate constants for substrate hydrolysis in the absence and presence of Na⁺. They reported ~10-fold increases in initial association and final deacylation rates, and interpreted this as indicating that 'The conformational change slow→fast must be such to drastically enhance the rate of substrate binding to the enzyme by widening the access to the catalytic pocket.' And, 'Access to the catalytic pocket opens up when Na⁺ is bound and remains in a wide open configuration during all steps of catalysis. As a result of this, substrate binding occurs on a faster time scale, and so do substrate dissociation and deacylation, which involve escape from the active site to the solution.' This appears to be true for all substrates, and when substrate or inhibitor affinity is significantly dependent on interactions in the aryl binding pocket, the effect is exaggerated. An example of this is the N-terminal peptide from hirudin which binds in a well characterised manner in the active site cleft, with Tyr3 occupying the aryl binding site (De Filippis *et al.*, 2005). The wild-type peptide binds with a 35-fold preference for the fast form, but when it is substituted for Ala, preference is reduced to 1.3-fold. Conversely, when the Tyr is substituted by larger, more hydrophobic synthetic groups, Bip and β-Nal, preference for the Na⁺-bound form increases to 60 and 85-fold, respectively. Thus, while access to the whole of the active site cleft is affected by Na⁺ binding, opening of the aryl binding pocket is of particular relevance. Interestingly, two of the hydrophobic clusters identified by De Filippis and colleagues (De Filippis *et al.*, 2005) (Tyr94, Trp96, Tyr60a, Pro60c, Trp60d and Val163, Cys168-Cys182, Trp215, Phe227, Tyr225) line the active site cleft and contribute to the aryl binding site (the contiguous S2 and S4 pockets). The same study also demonstrated a rigidification of the γ-loop in response to Na⁺ binding, as indicated by a 2–3-fold reduction in rate of cleavage of the Ala147c-Asn147d bond by subtilisin.

Although the change in conformation of the active site cleft is sufficient to account for the altered rates of hydrolysis and improved inhibitor binding, other regions are allosterically linked. Obviously, the Na⁺ binding site itself is crucial for Na⁺ binding; mutations of the residues involved directly in Na⁺ coordination or indirectly involved in stabilizing the Na⁺ binding loop through hydrogen bonding or ionic interactions all result in catalytically deficient variants which resemble slow thrombin (see the following section). Thus, as one might expect, mutations in the Na⁺ binding site affect Na⁺ affinity by either favouring the slow form or disfavoring the fast form. This energetic linkage between two distinct parts of thrombin (the Na⁺ binding site and the active site) is the basis of thrombin allostery. Interestingly, linkage has also been observed for exosite I of thrombin with the active site cleft and the Na⁺ binding site (Ayala and Di Cera, 1994). Binding of an exosite I directed peptide from the C-terminus of hirudin (known as hirugen) causes the same changes to the kinetics of chromogenic substrate cleavage as does Na⁺ binding; namely, an increase in rates of substrate association and product dissociation, with no further increase provided by the additional binding of Na⁺. Conversely, there is no effect of hirugen on substrate hydrolysis if Na⁺ is already bound. As one might expect, this linkage is also expressed in improved affinity for exosite I ligands when Na⁺ is coordinated, and improved Na⁺ binding affinity when exosite I ligands are bound. Fluorescence and CD changes are also identical for exosite I ligands and Na⁺, indicating that binding at either site results in the same conformational change (Ayala and Di Cera, 1994).

Which residues are involved?

The study of thrombin allostery has benefited greatly from the development of methods in site-directed mutagenesis and protein expression. There are multiple reports describing the properties of targeted thrombin variants, and even one that characterized a library of 78 Ala mutants. However, care must be taken when assessing the effect of mutations on the allosteric mechanism of thrombin. For example, a mutation on the 60-loop might affect substrate hydrolysis by altering the slow-fast equilibrium or by disrupting direct interactions with the

substrate. Even changes in charged residues outside of the active site cleft might affect the rate of hydrolysis by altering electrostatic steering. In addition, any mutation which affects the stability of thrombin is likely to have an influence on its activity. Although interpretation of the effects can be problematic, much has been learnt from studies on thrombin variants. In this review I will highlight the most important studies, and rank the mutants by the degree to which Na^+ affinity is altered (normally reduced). The selected list of implicated residues is given in Table 1.

The first site-directed mutagenesis study identified Glu39, Trp60d, Glu192, Asp221 and Asp222 as comprising part of the 'allosteric core' of thrombin based on activity in the presence and absence of Na^+ (Guinto *et al.*, 1995). Although it was disappointing that K_{d} s for Na^+ were not determined, it was interesting that the three regions to which these residues belong are the Na^+ binding site, the active site and exosite I. Mutation of each of the Na^+ -coordinating residues, Arg221a and Lys224, to Ala reduced the affinity for Na^+ by about 5-times due to the breaking of ionic interactions which stabilize the fast form; Arg221a makes a salt-bridge to Glu146, and Lys224 makes a hydrogen bond to the main chain of Ser171 and a salt-bridge to Glu217 (Dang *et al.*, 1997). Not unsurprisingly, mutation of the salt-bridging partners also had a significant effect on the ability of thrombin to bind Na^+ (Dang *et al.*, 1997; Pineda *et al.*, 2004).

The 186-loop is adjacent to the Na^+ binding loop and has also been implicated in thrombin allostery. In an attempt to activate thrombin by substituting the amino group of a Lys side chain for the Na^+ ion, Roy and colleagues (Roy *et al.*, 2001) mutated Gly184 to Lys. Instead of the super thrombin they hoped to create, the mutation resulted in a 200-fold reduction in activity and knocked out Na^+ binding. While mutations of residues 186 through 186d had only modest effects on the activity of thrombin, deletion of the whole loop reduced activity by 3,700-times and Na^+ affinity by 7-times (Prasad *et al.*, 2003). Arg187 makes multiple contacts with Asp221 and Asp222, and its mutation to Ala reduced affinity for Na^+ by 15-fold (Prasad *et al.*, 2003). That the conformation of the Na^+ binding loop was critical for Na^+ coordination was further enforced by the observation that mutation of Tyr225 to Pro abrogated Na^+ binding (Dang and Di Cera, 1996). The authors concluded that Tyr at 225 is the evolutionary hallmark of Na^+ binding proteases, and that the mutation to Pro effectively stabilised the slow conformation. One residue which links the Na^+ binding site to the aryl binding pocket was also identified as crucially involved in thrombin allostery; when Trp215 was mutated to Phe there was no effect on the activity of thrombin or affinity for Na^+ , but the fluorescence enhancement which usually reports Na^+ binding was abolished (Arosio *et al.*, 2000). The Trp215 to Ala mutant was ~9-fold deficient in Na^+ binding, and the Tyr mutant was 4-fold deficient (Arosio *et al.*, 2000), consistent with the energetic cost of burying a hydroxyl group during the slow→fast transition (Huntington and Esmon, 2003). The authors concluded that the side chain of Trp215 must change environment upon Na^+ binding from a solvent exposed state to a buried state, to account for the associated fluorescence change. The link between the active site and the Na^+ binding site was also noted, stating that 'perturbation of residue 215 propagates to the neighbour residues G216 and E217, producing changes in the access to the S1 site and reduced Na^+ binding.' One other tryptophan residue, Trp141, was later shown by the same group to contribute significantly to the Na^+ induced fluorescence enhancement (Bah *et al.*, 2006). The disulfide bond which links the active site to the Na^+ binding site, Cys191-Cys220, appears to play a role in transmission of conformational change, as the Ala mutations totally abrogate the fluorescence change, although Na^+ still enhances the rate of peptide substrate cleavage by ~10-fold (Bush-Pelc *et al.*, 2007). By far the most thorough approach to identifying the residues involved in the Na^+ induced conformational change is described in the recent report by Pineda and colleagues (Pineda *et al.*, 2004), where 78 Ala mutants were characterized for Na^+ binding affinity and substrate hydrolysis activity. They arbitrarily used a cut-off of 1.5 log units change in Na^+ affinity to define the residues comprising the 'allosteric core' of thrombin: Asp189,

Glu217, Asp222 and Tyr225. This would mean that only residues which, when substituted by Ala, reduced affinity by over 31-times can be considered important to thrombin allostery! A more inclusive approach is to grade the importance of the 78 residues according to the degree to which Na⁺ affinity was affected by the Ala mutations (Table 1). The majority of the substitutions resulted in less than a 1.8-fold (0.25 log) difference in Na⁺ affinity, and are unimportant in Na⁺ allostery. Residues whose Ala substitutions resulted in a 0.25 to 0.5 log unit effect are considered moderately important; 0.5 to 0.75 log unit effects are of medium importance; 0.75 to 1 log unit effects are of medium-high importance; 1 to 1.5 log effects are of high importance; and above 1.5 log reductions in affinity indicate residues of extreme importance. The residues that fall between moderate and extreme, and those whose substitutions (often to residues other than Ala) resulted in large effects on Na⁺ affinity or activation (labelled as 'Others' in the table) are considered to be involved in the Na⁺ induced conformational change, and are listed in Table 1. It is interesting to note that some of the residues identified in earlier studies as being part of the allosteric core, were down graded in importance when their Ala mutants were characterised as part of the 78 residue library. Thus, Glu39 is no longer part of the 'allosteric core' and is now considered unimportant to Na⁺ binding, and Trp60d is now only moderately important instead of playing a 'critical role' (Guinto and Di Cera, 1997).

Figure 3A shows all of the residues in Table 1 plotted on the structure of thrombin. Several important points are immediately clear. Firstly, the important residues are clustered and do not extend over the entire surface of thrombin. We can therefore expect that the conformational changes induced by Na⁺ binding will be limited to certain distinct regions of thrombin and will not affect the secondary structure content or tertiary structure. This is consistent with the interpretation of the far UV CD spectra, discussed above. Secondly, the regions identified by mutagenesis are the same as those highlighted by functional studies on wild-type thrombin, namely the Na⁺ binding site, the active site and exosite I. Mutagenesis allowed the additional identification of residues in between these three regions, and these residues would be in position to aide in the observed energetic coupling between the sites. While an exhaustive list of possible mutations has not been evaluated, for example residues at the stem of the γ -loop have not been studied, sufficient numbers of non-perturbing mutations have been examined to conclude that the clustering apparent in Figure 3A accurately reflects the regions involved in Na⁺ binding and the ensuing conformational change.

Crystallographic structures of slow and fast thrombin

So, how does the biochemical and mutagenesis data collected over the last three decades fit with the published structures of thrombin? Well, there is general consensus that the vast majority of the 200+ structures of thrombin deposited in the Protein Data Bank (PDB) are of thrombin in its fast form, whether or not Na⁺ is seen to be coordinated. This is because, until very recently, thrombin was always crystallised in the presence of an active site or exosite I inhibitor. Since we know that the two regions are energetically coupled with the Na⁺ binding site, the absence or presence of Na⁺ in the crystallisation conditions would be irrelevant (however, most of these structures are from crystals grown in the presence of Na⁺). This is nicely illustrated by the study which originally identified the Na⁺ binding site (Zhang and Tulinsky, 1997). Zhang and Tulinsky produced crystals of thrombin bound to the exosite I binding peptide hirugen in the presence of over 500mM Na⁺. In order to determine the conformation of the slow form, the Na⁺ was slowly soaked out of the crystal to a concentration of about 8nM. As this is well below the known room temperature K_d of 25mM the authors expected to obtain a crystal structure of the slow form. However, although Na⁺ was absent in the resulting structure, 'the remainder of the thrombin structure was, unexpectedly, the same', and this was attributed to crystal packing effects. It was therefore clear that in order to obtain a crystallographic structure of thrombin in its slow form it was not enough to remove Na⁺;

crystals would have to be grown in the absence of Na⁺ and active site or exosite I binding ligands or inhibitors. Even then, care would be required when interpreting the resulting structure since crystal contacts themselves typically make a free energy contribution of 3–6 kcal/mol (Drenth and Haas, 1992), significantly more than the free energy difference between the slow and fast forms (~2.2 kcal/mol).

Over the last 5 years, several crystal structures of the putative slow form of thrombin have been deposited in the PDB. These are from crystals of wild-type thrombin (or S195A, D102N or R77aA variants) grown in the absence of Na⁺ and ligands/inhibitors, or of thrombin variants with perturbed Na⁺ binding (e.g. E217K), possibly representing recombinant slow forms. In order to determine where and to what degree these structures differ from fast thrombin and from one another, the structures have been trimmed down to remove inherently flexible regions. Thus, C α root mean square deviations (RMSD's) were calculated for residues 16–142 and 153–244 of the heavy chain for the various thrombin structures, and the results are listed in Table 2. In order to assess what RMSD value represents a real conformational change, it is first necessary to get an idea of how structures that are acknowledged to be in the fast form differ from one another. Five structures of fast thrombin were chosen: 1PPB is the original thrombin structure of Wolfram Bode and colleagues (Bode *et al.*, 1989), and was grown in the presence of Na⁺ with an active site inhibitor (PPACK) bound; 1HAH is a structure of active site free thrombin (Vijayalakshmi *et al.*, 1994), grown in the presence of Na⁺ and bound to hirugen at exosite I; 1HXF is isomorphous to 1HAH and has hirugen bound, but Na⁺ has been soaked out (Zhang and Tulinsky, 1997); 1SG8 has two molecules of R77aA thrombin in the asymmetric unit (chains B and E), and crystals were grown in the presence of Na⁺, but in the absence of ligand or inhibitor (Pineda *et al.*, 2004). When these structures are superimposed on 1PPB, an average C α RMSD value of 0.39 $\pm$ 0.04 Å is obtained. Thus, thrombin structures that are acknowledged to be in the identical fast conformation typically have RMSD values around 0.4Å when compared to 1PPB. This value is also obtained when the two copies of fast thrombin in 1SG8 are compared to each other (0.35Å).

When the crystallographic candidates for the slow form are compared to fast thrombin, two classes emerge: one where the conformational difference is similar to that found between the fast forms (RMSD 0.41 $\pm$ 0.04Å for the two copies from 1SGI (Pineda *et al.*, 2004)); and, a second where the conformational difference is around three-times that of class I (RMSD 1.25 $\pm$ 0.04). If we consider identical fast conformations have an average RMSD of 0.4Å, then this can be considered the 'noise' level, and significance of conformational change can be defined in terms of signal-to-noise ratio (SNR = x/y, where x is the average RMSD of a putative slow form and y is the average RMSD of the fast controls, when compared to 1PPB). This has been calculated on a per residue basis, and the resulting bar graph is shown in Figure 4 for the two classes of putative slow forms. For the first class (1SGI), only five residues undergo a significant (SNR greater than 2) change in C α position (G43, N60g, R73, E186b and G216), and only one of these (Arg73) shows a 3-fold difference. The difference between the first and second class of putative slow structures is striking, with a large subset of residues in class II undergoing significant movement relative to the fast controls. When the C α SNR is plotted on the structure of thrombin, it is evident that the regions of the class II structures that significantly change conformation correspond to those identified by biochemical and mutagenesis studies (Figure 3B), namely the Na⁺ binding site, the active site and exosite I. In order to determine which of the putative slow structures exhibited conformational changes in the residues identified in Table 1, the main chain and side chain RMSD SNRs were plotted for each structure relative to the fast controls (Figure 5). In contrast to class II, the class I structures showed no significant main chain conformational difference relative to fast thrombin, and only a few residues (60d, 73, 163, 191, 192, 221a, and 233) showed significant side chain movement. These data reinforce what was clear from the average C α RMSD values, namely, that the putative slow structures of 1SGI are conformationally indistinguishable from fast thrombin.

The four members of class II were consistently different from the fast controls for most of the residues identified by mutagenesis studies.

It is not possible to judge by any objective measure which of the class II candidates, 1RD3 (Carter *et al.*, 2004), 2AFQ (Johnson *et al.*, 2005) or 2GP9 (Pineda *et al.*, 2006), is the structure of slow thrombin; that is to say, the crystal structure which best represents the conformation of slow thrombin in solution. All of them show significant differences in regions known to be involved in the Na⁺ activation of thrombin, in particular: the Na⁺ binding loop, from residue 215 to 224; and the contiguous loop from 184 to 193, stretching from the 186 loop to the active site loop. Interestingly, these loops are fully modelled in the class II crystal structures and are therefore in an ordered state distinct from that of the fast form. A shared feature of functional importance is the observed movement of the entire Na⁺ binding loop towards the active site cleft. This has ramifications for the catalytic activity of slow thrombin. For instance, the S1 pocket is blocked in all structures by the newly positioned Na⁺ binding loop (Figure 6A). Another feature shared by these structures is a reorganisation of the aryl binding pocket. In 2GP9, Trp215 adopts a conformation which would block P2 and P4 interactions, whereas in 1RD3, Trp215 protrudes only slightly into the P4 pocket (Figure 6A). In addition, all of the class II structures reveal the destruction of the oxyanion hole through a flipping of Gly193, and the concomitant flipping of the adjacent main chain of Glu192 results in non-catalytic hydrogen bonding with Ser195. An example of this is shown in Figure 6B for 1RD3, but similar non-catalytic H-bonding is also seen for the other class II structures. The shared features of these structures provide a structural explanation for the biochemical observations that the active site, in particular the S1 and aryl binding pocket, opens up to become more accessible to substrates and inhibitors when Na⁺ is bound. The class II structures are thus likely to represent the slow form of thrombin.

What is the allosteric mechanism of thrombin?

One of the surprise features of the class II structures is that the loops involved in conformational change are not disordered, but are seen to exist in states stabilised by networks of hydrogen bonds distinct from those sampled in the fast state. This suggests that the slow and fast forms represent energetic minima in solution. Since all of the class II structures revealed blockage of the S1 pocket and non-catalytic hydrogen bonding in the active site, it can be concluded that that conformational changes must take place before a peptide substrate could be hydrolyzed. One might conclude that the slow form would therefore be inactive. How can this be reconciled with the fact that thrombin in the absence of Na⁺ still has appreciable catalytic activity? The answer to this question is that thrombin exists in a rapid and dynamic equilibrium between inert and active states, and that Na⁺ binding merely stabilises the active conformation. This has been established by recent rapid kinetics studies.

Jules Shafer and colleagues (Lai *et al.*, 1997) studied the binding of Na⁺ to thrombin by following intrinsic fluorescence. They observed biphasic binding, with a fast phase within the dead time of the stopped-flow device, and a slow phase of $\sim 30 \text{ s}^{-1}$ which was independent of Na⁺ concentration. The rate constant of the slow phase was, however, dependent on temperature ($\sim 200 \text{ s}^{-1}$ at 25°C and $\sim 450 \text{ s}^{-1}$ at 37°C). Interestingly, the Na⁺ binding is still biphasic at higher temperature and the ratio of fast to slow phases is 'similar' to what was obtained at 5°C. The same biphasic behaviour was observed when the binding of an active site inhibitor or an exosite I directed peptide was monitored by change in intrinsic fluorescence, and the same slow rate of $\sim 30 \text{ s}^{-1}$ was obtained. The data fit a model where Na⁺-free thrombin exists in an equilibrium between two forms, one incapable of binding Na⁺ (or active site inhibitors or exosite I peptides) and another that is binding competent, and the conversion from the inactive to active forms accounts for the temperature-dependent slow phase. These conclusions were supported by a similar study published in 2006 (Bah *et al.*, 2006). A year

later, Gianni and colleagues (Gianni *et al.*, 2007), using ultra rapid kinetics techniques, were able to resolve a linear dependence of the fast phase on Na⁺ concentration, indicating that the conformational change reported by fluorescence actually *precedes* Na⁺ binding. In other words, Na⁺ binding does not *induce* a conformational change, rather, Na⁺ binding stabilises one of the equilibrium conformations of thrombin. The observation simplifies the scheme that describes Na⁺ binding to thrombin:

Scheme 1

where E* is in a conformation incapable of binding to Na⁺ or active site ligands; E is a distinct conformer which is binding competent; and E:Na⁺ is the Na⁺-stabilised form of E. The authors conclude that ‘The interconversion of E and E* that precedes Na⁺ binding is the only conformational transition in Scheme 1.’ We can quite easily fit this scheme to the three classes of structures described in the previous sections; the fast controls which have Na⁺ bound (e.g. 1SG8) represent E:Na⁺; the Na⁺-free structures which are conformationally indistinguishable from E:Na⁺ (e.g. 1SGI and 1HXF) represent E; and the class II structures (1RD3, 2GP9 and 2AFQ) represent E*. The equilibrium position between E* and E determines the observed activity of thrombin, and the role of Na⁺ is simply to stabilise the E state. The ratio of E* to E in the absence of Na⁺ is approximately 1:1 (Gianni *et al.*, 2007), and is likely to be independent of temperature (Lai *et al.*, 1997).

Summary

Thrombin is the central protease in the blood coagulation cascade and plays crucial functions in promoting and down regulating its own formation. Thus, any factor which alters the activity of thrombin is potentially relevant to human health and disease. The binding of Na⁺ to thrombin is specific and increases the rate at which thrombin cleaves most substrates, including several of its haemostatic targets. In this review I summarised the data collected over the last thirty years by several groups around the world. It was necessary to focus on what I considered to be the most relevant work, and an attempt was made to focus on the data and not the interpretations. The biochemical work summarised in the early part of the review unequivocally indicates that the binding of Na⁺ to thrombin induces a conformational change affecting specific regions in thrombin. Of particular relevance is the opening of the active site cleft, which easily accounts for the general activation of thrombin. This has been demonstrated by many groups using several different probes for active site accessibility, and thus a closed active site must be considered a requisite feature of any crystal structure claiming to represent slow thrombin. However, there has been some confusion caused by the interpretation of the structures 1MH0 and 1SGI (same structures at different resolution) as slow thrombin, since there was no significant conformational change relative to acknowledged fast forms. It was argued that a water channel connecting the Na⁺ site and the S1 pocket and a 1Å movement of the Oy of Ser195 accounted for the observed decreases in K_m and increases in k_{cat} upon Na⁺ binding. However, the fact that exosite I binding peptides recapitulate the Na⁺ effect disproves the water channel theory, and the tiny movement of the side chain of Ser195 is consistent with the normal positional variation of this residue in the many crystal structures of thrombin. Indeed, when compared to the Oy position of 1HAH, the four copies of ‘slow’ thrombin from 1MH0 and 1SGI move by 0.98, 0.54, 1.03, 0.82 Å and the acknowledged fast form 1HXF moves by 0.85Å. Needless to say, neither of these two problematic explanations addresses the accessibility of the active site cleft, and can thus be discounted out of hand. The structures which best account for the biochemical data are those of class II (1RD3, 2GP9 and 2AFQ). They consistently show a closure of the active site cleft and non-catalytic hydrogen bonding.

These features would render thrombin inactive, but for the fact that this form is in rapid equilibrium with an active form, where the active site has opened and the catalytic residues are in the correct position. The rapid kinetics data showed that the only conformational change occurs during this equilibrium step, with Na⁺ binding merely stabilising the active state. What remains to be determined is what the populations of these three states are under physiological conditions. If, as suggested, E* represents only a minor species (Bah *et al.*, 2006), then there is unlikely to be any physiological relevance to the *in vitro* observation that Na⁺ binding activates thrombin. Further study is required to determine whether Na⁺ allostery has been a big waste of time or is the next big thing!

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Figure 1.

A schematic of the major functions of thrombin in haemostasis. Solid arrows represent procoagulant activities and dashed arrows are for anticoagulant functions. The thick arrows illustrate how the tenase and prothrombinase complexes boost thrombin generation from prothrombin. Some cofactors for thrombin activity are indicated along the arrows.

Figure 2.

The structural features of thrombin. (A) A ribbon diagram of thrombin (from 1JOU), coloured from N to C terminus of the heavy chain (blue to red) with the light chain in grey, is shown in the standard orientation (A, B and C). The active site cleft is facing so that a substrate would run from left to right, with the P residues (N-terminal of scissile bond) on the left and the P' residues (C-terminal of the scissile bond) on the right (as in B). The side chains of the catalytic residues are shown as rods, and the N atoms of the oxyanion hole are shown as small blue spheres. The 60 and γ -loops are indicated by cyan and yellow spheres (residues 60g and 147d), respectively, and Na^+ is shown as a purple sphere. The loops encompassing the Na^+ ion are the 186-loop underneath and the 220-loop above. Disulphide bonds are shown as rods. (B) A

close-up of the active site cleft with a substrate peptide bound (magenta rods for residues 390 to 395 of AT, from 1TB6) illustrates the precise alignment of the catalytic residues and the scissile (P1-P1') bond (hydrogen bonding shown as broken red lines). (C) The surface representation of the electrostatic properties of thrombin demonstrates the depth of the active site cleft (peptide substrate as in B) and the positions of the two anion binding exosites. (D) The Na⁺ binding site of thrombin and its connection to the catalytic site is shown. Na⁺ coordinates the main chain oxygens of Arg221a and Lys224 and four water molecules in an octahedral fashion. The Na⁺ binding loop (residues from 215 to 225 shown) is directly connected to the active site (His57, Asp102 and residues 191 to 195 are shown) via a disulphide bond between Cys220 and Cys191. Many of the residues known to be involved in Na⁺ binding and the conformational change are found in this region.

A

B

Figure 3.

Stereo representations of thrombin highlight the regions implicated in thrombin allostery by mutagenesis (A) and structural (B) studies. (A) A ribbon diagram of thrombin in the standard orientation is coloured grey, with the residues implicated by mutagenesis studies coloured according to importance, as in Table 1 (Na⁺ shown as the purple sphere). The stereo view reveals the evident clustering of important residues in the Na⁺ binding site, the active site, exosite I and intervening regions. (B) A ribbon diagram of the heavy chain of thrombin (residues 16–142, 153–244) is coloured to illustrate the regions which change conformation when class II slow structures are compared to the fast controls. The average signal to noise ratio (SNR, as defined in the text) for all class II structures relative to all fast controls is coloured

from cyan to red (cyan for SNR less than 2, yellow for SNR between 2 and 3, orange for SNR between 3 and 4 and red for SNR greater than 4).

Figure 4.

A bar graph comparing Cα SNR for class I (black) and class II (red) putative slow forms relative to the fast controls. A per residue average Cα RMSD for each class is divided by the average RMSD for that residue among the fast controls. The resulting signal to noise ratio (SNR) is plotted here for all of the residues used for comparison (16–142, 153–244). SNR greater than 2 is considered to represent significant conformational change and is indicated by the horizontal line.

Figure 5.

Bar graph comparing $C\alpha$ (A) and side chain (B) RMSDs for each putative slow structure to the fast controls for the residues identified by mutagenesis. Most residues identified by mutagenesis undergoes significant main chain and side chain movement for class II slow structures (red, 2AFQ, 2GP9, 1RD3), but not for class I structures (black, two copies from 1SGI), with few exceptions.

A

B

Figure 6.

The functional consequences of Na⁺ binding include an opening of the active site cleft and formation of the catalytic site. (A) A stereo view of the active site cleft of thrombin (1PPB, semitransparent surface), with slow structures from class II superimposed (1RD3 cyan, 2AFQ green, and 2GP9 magenta), reveals an obstruction of the S1 pocket and a partial blocking of the aryl binding site (S4, especially for 2GP9). (B) The catalytic site of a representative slow structure (1RD3, cyan) is compared to that of the fast control (1PPB, yellow). In class II structures, Glu192 and Gly193 flip, engendering non-catalytic hydrogen bonding (broken red lines) and destroying the oxyanion hole.

Table 1

Residues involved in the Na⁺ activation of thrombin, graded according to the effect of the indicated mutation on Na⁺ affinity. Residues in the ‘Other’ category are included based on criteria referred to in the text. The colour coding is preserved in Figure 3A.

Moderate (green)	Medium (cyan)	Med-high (yellow)	High (orange)	Extreme (red)	Other (blue)
Y60aA	E192A	V163A	T172A	D189A	W141F
P60bA	R221aA	G193A	Y184aA	E217A	E146
P60cA	F227A	W215A	R187A	D222A	G184K,R
W60dA		K224A	S214A	Y225A	C191A-C220A
F60hA		G226A	G223A		
R73A					
E80A					
R233A					

Table 2

Structural comparison of acknowledged ‘fast’ forms and putative ‘slow’ forms relative to the control structure 1PPB. C α RMSDs for heavy chain residues 16–142 and 153–244 (241 atoms) were calculated for the structures indicated. Some putative slow forms were not included in the analysis because residues in either the 186 or 220-loops were not modelled (2AFQ_D, 2A0Q and 1TQ0).

Fast Controls (resolution)	Thrombin	C α RMSD vs. 1PPB
1PPB (1.9Å)	PPACK inhibited	-
1HAH (2.3Å)	Na ⁺ and hirugen bound	0.35
1HXF (2.1Å)	hirugen bound, Na ⁺ -free	0.37
1SG8_B (2.3Å)	Na ⁺ bound, R77aA	0.40
1SG8_E (2.3Å)	Na ⁺ bound, R77aA	0.45
Putative Slow Forms		
Class I		
1SGI_B (2.3Å)	Na ⁺ free form of 1SG8	0.40
1SGI_E (2.3Å)	Na ⁺ free form of 1SG8	0.37
Class II		
2AFQ_B (1.9Å)	Na ⁺ free, wild-type	1.30
2GP9 (1.9Å)	Salt-free, D102N	1.27
1RD3_B (2.5Å)	E217K	1.25
1RD3_D (2.5Å)	E217K	1.21