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Review

Molecular mechanisms of thrombin function

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Abstract. The discovery of thrombin as a Na^+-dependent allosteric enzyme has revealed a novel strategy for regulating protease activity and specificity. The allosteric nature of this enzyme influences all its physiologically important interactions and rationalizes a large body of structural and functional information. For the first time, a coherent mechanistic framework is available for understanding how thrombin interacts with fibrinogen, thrombomodulin and protein C, and how Na^+ binding influences the specificity sites of the enzyme. This information can be used for engineering thrombin mutants with selective specificity towards protein C and for the rational design of potent active site inhibitors. Thrombin also serves as a paradigm for allosteric proteases. Elucidation of the molecular basis of the Na^+-dependent allosteric regulation of catalytic activity, based on the residue present at position 225, provides unprecedented insights into the function and evolution of serine proteases. This mechanism represents one of the simplest and most important structure-function correlations ever reported for enzymes in general. All vitamin K-dependent proteases and some complement factors are subject to the Na^+-dependent regulation discovered for thrombin. Na^+ is therefore a key factor in the activation of zymogens in the coagulation and complement systems.

Key words. Allosteric enzymes; blood coagulation; complement; molecular evolution; monovalent cations; protein engineering; serine proteases; thrombin.

Introduction

Serine proteases of the chymotrypsin family [1, 2] participate in key physiological functions like digestion, blood coagulation, fibrinolysis and complement [3]. Proteases involved in digestive processes, like trypsin, have wide specificity and are also found in organisms as primitive as archaea. In contrast, proteases involved in the more specialized functions of blood coagulation, fibrinolysis and complement have narrow specificity and are found exclusively in vertebrates [4–6].

The molecular origin of protease specificity, its regulation and evolutionary development remain mostly elusive. Elucidation of these aspects is crucial for structure-function correlations and the development of more specific and potent active site inhibitors for pharmacological purposes. The recent discovery of the Na^+-induced allostery in thrombin [7] has provided a coherent mechanistic framework to understand the complex function of this enzyme. This phenomenon has also revealed important new aspects of serine protease function, particularly how some of the more evolved enzymes have developed additional mechanisms to control catalytic activity and specificity [8]. Both of these functions can now be engineered rationally by altering Na^+ binding [9].

A detailed account of these developments is offered in this review, which summarizes the molecular mechanisms of thrombin interaction with its key physiological ligands and puts thrombin allostery in the context of serine protease function and evolution. The new picture emerging for thrombin is that of a remarkably articulate enzyme that has exploited allostery to accomplish

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Table 1. Site of cleavage (↓) by thrombin on natural substrates.

Substrate	Sequence	Reference
Fibrinogen (Aα chain)	FLAEGGGVR↓GPRVVERH	[28]
Fibrinogen (Bβ chain)	NEEGFFSAR↓GHRPLDK	[28]
Factor XIII	TVELEGVPR↓GVNLQQ	[29]
Factor VIII	LSNNAIGPR↓SFSQNSRHP	[30]
Factor V	RLAAALGIR↓SFRNSSLNQ	[31]
Factor VII	RNASKPQGR↓IVGGKVCPK	[32]
Thrombin receptor 1	ATNATLDPR↓SFLLRNPND	[17]
Thrombin receptor 2	LAKPTLPIK↓TFRGAPPNS	[19]
Protein C	NQGDQVDPR↓LIDGKMTRR	[33]

its multiple roles in hemostasis. Thrombin regulation now serves as a paradigm for allosteric serine proteases in general and reveals an underlying complexity for this class of enzymes.

Thrombin structure

The coagulation system was originally imagined as a cascade, a waterfall of enzymatic events with each protease being responsible for a specific reaction [10, 11]. It has now become apparent that the cascade is an intricate network of reactions, where each protease accomplishes diverse and even opposing functions [12–15]. Thrombin demonstrates this complexity best, in so far as it is capable of two important and opposite roles. One role, procoagulant, entails the conversion of fibrinogen into the insoluble fibrin clot, the promotion of platelet aggregation, the stabilization of the ensuing clot by activation of factor XIII, and the feedback enhancement of its own generation from prothrombin by activation of factors V, VIII and XI. A second role, anticoagulant, involves the thrombomodulin-assisted conversion of protein C into an active component that cleaves and inactivates factor Va together with protein S, thereby limiting the conversion of prothrombin into thrombin (catalysed by the prothrombinase complex) [16]. In addition to its primary roles in coagulation, thrombin has a variety of important effects on a number of cell lines upon binding to its receptors [17–19]. In the blood, thrombin has a short life of only a few minutes due to scavenging by antithrombin III [20–22], which rapidly forms an inactive adduct with the protease in the presence of heparin. All thrombin interactions are affected by the equilibrium between the slow and fast forms [7]. The slow → fast transition is triggered by the binding of Na^+ and results in enhanced specificity towards a variety of synthetic and physiological ligands. The Na^+-bound fast form has higher specificity than the Na^+-free slow form towards fibrinogen, the thrombin receptors, thrombomodulin and antithrombin III, whereas the slow form is more specific towards protein C [23].

Thrombin is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) residues that are covalently linked through a disulfide bond [24]. The B chain carries the functional epitopes of the enzyme and has the typical fold of serine proteases [25], with two six-stranded β-barrels of similar structure that pack together asymmetrically to accommodate the residues of the catalytic triad H57, D102 and S195* at their interface (fig. 1). The catalytic triad polarizes the side chain of the active site S195 for a nucleophilic attack on the C atom in the scissile bond of the substrate. The C atom is converted into a tetrahedral intermediate in the transition state, which is stabilized by hydrogen bonds between the charged carbonyl O atom of the peptide group of the scissile bond and the amide hydrogen atoms of G193 and S195 which form the oxyanion hole. The substrate is then acylated by the O^{γ} atom of S195 after transfer of a proton to H57 and its C-terminal fragment is released. Nucleophilic attack by a water molecule catalyses deacylation, releasing the carboxylic acid product and the N terminal fragment of the substrate, which restores the state of the catalytic triad. D102 anchors H57 in the correct orientation for proton transfer from and to S195, which compensates for the developing positive charge [27].

A list of natural substrates for thrombin is given in table 1. Except for the consensus Arg at P1**, (with the exception of the newly discovered receptor carrying a

* The numbering refers to chymotypsin. Insertions relative to chymotrypsin are denoted by a letter in lower case following the redidue number (e.g. R221a) to avoid confusion with single-site mutations.

** The notation is that of Schechter and Berger [34]. Residues of the substrate interacting with the enzyme are labeled with a P and a number from 1 to *N*, starting from the scissile bond and moving to the N terminus. Residues of the enzyme making contacts with the substrate are called *specificity sites* and are labeled with an S. The amino acid at P1 of the substrate makes contacts with the specificity site S1 of the enzyme, P2 contacts S2 and so forth. The P residues of the substrate are necessarily contiguous in sequence, whereas this restriction does not apply to the S residues of the enzyme. Residues on the C-terminal portion of the scissile bond of the substrate are numbered P1', P2' and so forth and the corresponding specificity sites on the enzyme are S1', S2' and so on. The scissile bond is positioned between P1 and P1'.

Lys at this position), there seems to be little conservation around the cleaved bond. The trypsin-like specificity for basic residues at P1 is conferred to thrombin by the presence of D189 at the S1 site occupying the bottom of the catalytic pocket. A striking difference emerges from the comparison of fibrinogen and protein C: this substrate carries Asp at P3 and P3′, where fibrinogen has Gly (P3) or basic (P3′) residues. This suggests that the S3 site of thrombin cannot be the same for fibrinogen and protein C, and that a conformational transition must take place when thrombin switches from being a procoagulant to an anticoagulant factor. The transition is linked to the release of Na^+ from its site, leading to the fast → slow conversion of the enzyme [9, 23].

Numerous insertions are present in thrombin, relative to trypsin and chymotrypsin, shaped as loops connecting β-strands in the B chain. Two such insertions shape and narrow the access to the active site. The W60d loop defines the upper rim of the active site and screens H57 and S195 from the solvent. The loop contains an insertion of nine residues, from Y60a to I60i, and protrudes into the solvent with the bulky side chain of W60d providing most of the steric hindrance. Deletion of the P60b-P60c-W60d segment impairs inhibition of thrombin by antithrombin III [35], whereas replacement of W60d alone with the less bulky Ala or Ser reduces the interaction with antithrombin III [36] or fibrinogen [37, 38]. The charge reversal K60fE reduces fibrinogen binding five-fold with minimal alteration of thrombomodulin binding or protein C activation [39]. The K60fA substitution elicits similar effects [40]. A function analogous to that of the W60d loop has been hypothesized for the autolysis loop which shapes the lower rim of the access to the active site. However, proteolytic cleavage of this loop produces no significant functional changes [41, 42]. The autolysis loop contains a five residue insertion from A149a to K149e. Swapping this loop with the homologous region of trypsin, which is devoid of the A149a-K149e insertion, has minor effects on the properties of thrombin [43]. Deletion of the five residue insertion is inconsequential on thrombin function, except for a notable three-fold increase of protein C activation, whereas deletion of the entire loop from E146 through K149e results in selective loss of fibrinogen clotting [44].

In between the two insertion loops, the access to the active site of the enzyme hosts E192 in its middle. Its negatively charged side chain is not compensated by hydrogen bonds or ion pair interactions with neighbor residues. In other serine proteases like trypsin this residue is a Gln. The uncompensated charge of E192 plays an important role in discriminating against substrates carrying acidic groups near the scissile bond, like protein C and the thrombin receptor 1 (table 1). The replacement E192Q results in a 20-fold enhancement of protein C cleavage, but only in the absence of thrombomodulin [45]. Likewise, replacement of D167 at P3 with Phe in protein C (table 1) results in enhanced cleavage by thrombin [46].

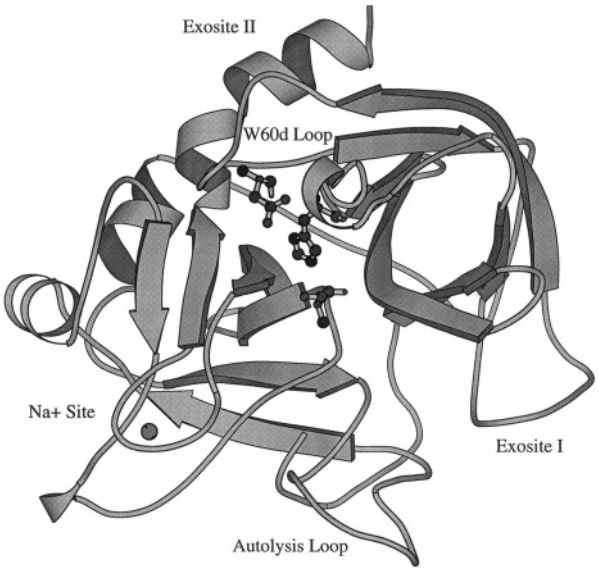

Figure 1. MOLSCRIPT [26] rendering of the B chain of human thrombin in the fast form. Residues of the catalytic triad are shown at the interface between the two β-barrel domains. The catalytic triad is located about 20 Å away from the bound Na^+ (ball). Important regions of the enzyme are noted.

The prominent loop centered around K70 is called exosite I and is homologous to the Ca^{2+} binding loop of the cognate proteases trypsin and chymotrypsin [47]. In these pancreatic proteases Ca^{2+} stabilizes the fold and confers increased resistance to proteolytic digestion. In thrombin the need for Ca^{2+} is eliminated by the insertion of K70, the side chain of which mimics the bound Ca^{2+} and obliterates the cavity available for binding this cation. In fact, thrombin does not bind Ca^{2+} up to mM concentrations [23]. A remarkable feature of the K70 loop is the conspicuous presence of positively charged amino acids, resulting in an intense positive electrostatic potential. The role of this potential is to provide electrostatic steering for fibrinogen, thrombomodulin, the natural inhibitor hirudin, and perhaps even the thrombin receptors to facilitate formation of a productive complex upon binding. Structural and functional data support exosite I as the dominant factor in recognition of fibrinogen [48–50], thrombomodulin [51, 52], hirudin [53–55] and the thrombin receptors [17, 19, 56, 57]. Although the epitopes for fibrinogen and hirudin binding overlap almost completely [37], the same is not true for thrombomodulin and fibrinogen. The mutation R73E affects both thrombomodulin and fibrinogen binding, but the mutant R75E compromises thrombomodulin binding more significantly [39].

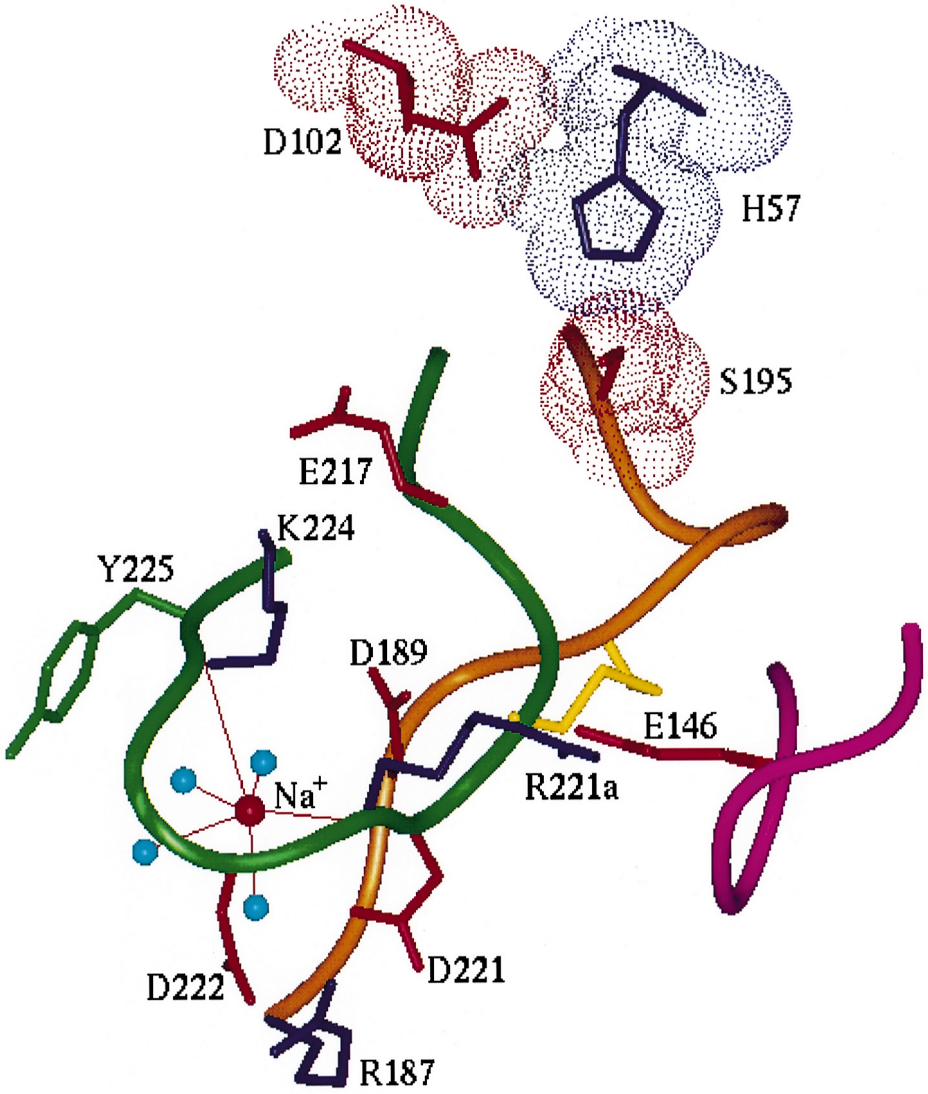

Figure 2. Na^+ binding environment of thrombin showing the backbone of residues 215–227, and the side chains of residues important for Na^+ binding and the catalytic activity of the enzyme. Residues of the catalytic triad, shown with their van der Waals surface, are located about 20 Å away from the bound Na^+. The side chain of D189 at S1 is 5 Å away from the Na^+, which is octahedrally coordinated by the carbonyl O atoms of K224 and R221a and four buried water molecules. Three ion pairs stabilize the Na^+ binding environment. R221a is ion-paired to E146 of the neighbor autolysis loop, K224 is ion-paired to E217 and R187 forms a bidentate ion pair with D221 and D222 flanking R221a. A disulfide bond between C220 and C191 (yellow) anchors the Na^+ binding loop to the neighbor segment 187–192 hosting D189 and E192. Mutation of Y225 to Pro perturbs the protein backbone and reorients the carbonyl O atom of K224 so that it is incompatible with Na^+ binding.

Exosite I also serves as an extended primed recognition site that can communicate changes to the catalytic moiety of the enzyme. Peptides derived from the physiological inhibitor heparin cofactor II [58], the thrombin receptor 1 [59] or the hirudin C-terminal domain [60] influence the active site of thrombin allosterically. The effect is however very small when thrombin is in the fast form, as also documented by the lack of significant structural changes [55]. Binding of hirudin C-terminal fragments or thrombomodulin to exosite I in fact promotes allosterically Na^+ binding and switches the enzyme from the slow to the fast form, so that the effect on catalytic activity and specificity is seen almost entirely and in a more significant manner when the enzyme is in the slow form [38, 61, 62].

On the other side of the enzyme, opposite exosite I, a prominent C-terminal helix hosts a number of posi-

tively charged residues that provide the anion binding exosite II. This is the site for interaction with polyanionic ligands like heparin [63, 64] and the chondroitin sulfate moiety of thrombomodulin [62, 65, 66]. The helix packs tightly against the domain supporting the catalytic D102 [25] and it is conceivable that binding to exosite II could influence allosterically the catalytic activity of the enzyme by affecting the position of the side chain of D102.

The Na^+ binding site (fig. 2) displays octahedral coordination, involving the carbonyl O atoms of R221a and K224 and four buried water molecules, tetrahedrally coordinated by protein atoms and other water molecules [67, 68]. Altogether these define a complex hydrogen-bonding network within the catalytic pocket [69]. Some of the hydrogen bonds in the network are conserved with trypsin [47]. Others are specific to thrombin and are associated with Na^+ and its coordination shell. The bound Na^+ is located 15–20 Å away from the catalytic triad and lies within 5 Å from D189 in the specificity site S1 with a water molecule mediating a hydrogen-bonding interaction with $O^{\delta 2}$ of D189. The Na^+ site lies within a cylindrical cavity formed by three antiparallel β-strands of the B chain (M180–Y184a, Y225–Y228, V213–C220), diagonally crossed by E188–E192 and shaped by the loop D221–K224 connecting the last two β-strands. The sequence C220–G226, involving the Na^+ binding loop and part of the last β-strand of the B chain, is almost completely conserved in thrombin from eleven different species, from hagfish to human [70], which supports the importance of Na^+ binding in thrombin function. The Na^+ loop is also present, with similar architecture, in all serine proteases of the chymotrypsin family [25] and plays a role in determining the specificity of trypsin and chymotrypsin [71, 72].

A crucial residue controlling Na^+ binding in thrombin and all other serine proteases of the chymotrypsin family is Y225 [8], the mutation of which to Pro abolishes Na^+ binding and produces a thrombin stabilized in the anticoagulant slow form that has enhanced specificity toward protein C [9]. The environment of the Na^+ site appears to be stabilized by three ion pairs. R221a is ion-paired to E146 of the autolysis loop, K224 is ion-paired to E217, and D221 and D222 form a bidentate ion pair with R187. When the bidentate ion pair is disrupted in the double mutant D221A/D222K, made to mimic the sequence found in factor Xa in the same region, thrombin assumes a conformation intermediate to the slow and fast forms. This has reduced activity toward fibrinogen but enhanced activity toward protein C [67]. The effects of disrupting the R221a-E146 ion pair are revealed by the properties of the natural mutant thrombin Salakta [73], E146A, which has a reduced clotting activity. The R221aA mutant displays similar properties due to reduced Na^+ binding [9]. Disruption of the K224-E217 ion-pair in the E217A mutant produces a 40-fold loss of clotting activity with only a two-fold reduction of protein C activation [74]. The K224A replacement produces similar effects due to reduced Na^+ binding [9].

The interaction with Na^+

Na^+ is the most important ligand of thrombin because of its ubiquitous presence in the physiological milieu where the enzyme functions in vivo. The fact that the Na^+ concentration is tightly controlled in the blood does not detract from the importance of Na^+ as the key effector of thrombin function. In fact, Na^+ is actively exchanged in the transition state upon binding of fibrinogen or protein C, just like the proton of the active site H57 is actively exchanged during catalysis even if the pH of the solution remains constant. Since fibrinogen binds to the fast form with higher affinity, it promotes the slow → fast conversion and Na^+ binding. On the other hand, binding of protein C promotes the fast → slow conversion and Na^+ release. Hence, the notion that a constant concentration of Na^+ in the blood must result

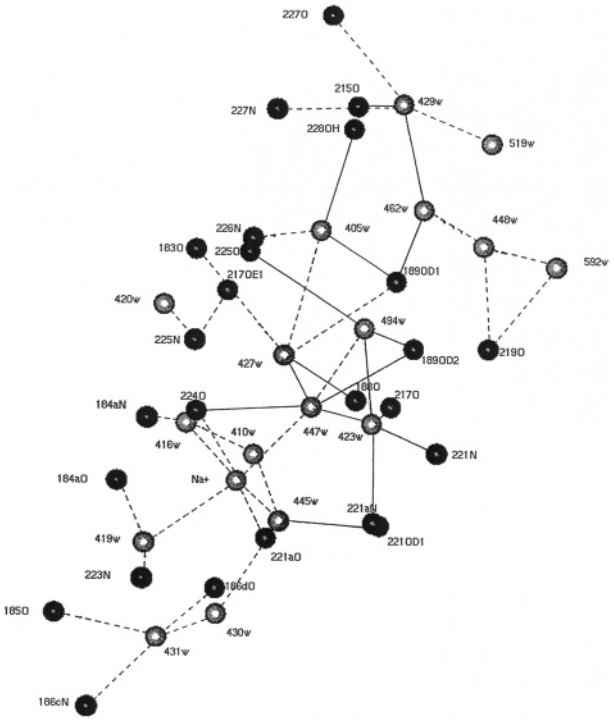

Figure 3. Molecular environment of the Na^+ binding site of thrombin and the conspicuous network of water molecules embedding the region. The hydrogen-bonding network involves the bound Na^+, 17 water molecules (gray circles) and several protein atoms (dark circles). Some hydrogen bonds (continuous lines) are conserved topologically with trypsin, which does not bind Na^+. Others (discontinuous lines) are specific to thrombin. The bound Na^+ is octahedrally coordinated by the carbonyl O atoms of K224 and R221a and four water molecules (419, 416, 445 and 447). The side chain of D189 is nearby, with water 447 mediating a contact between $O^{\delta 2}$ of D189 and the bound Na^+.

Table 2. Properties of the slow and fast forms of thrombin under physiological conditions[a].

	Slow form	Fast form	[NaCl] = 145 mM	r^b
Fibrinopeptide A release k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	1.5 ± 0.1	35 ± 4	17 ± 1	23
Fibrinopeptide B release k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.73 ± 0.04	17 ± 1	9.4 ± 0.5	23
Thrombin Receptor 1 k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	1.4 ± 0.1	54 ± 2	31 ± 1	39
Protein C activation[c] k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.32 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.7
Thrombomodulin binding K_d (nM)	5.3 ± 0.7	0.60 ± 0.02	0.99 ± 0.04	9
Antithrombin III inhibition[d] k_{on} ($\mu M^{-1}s^{-1}$)	4.0 ± 0.2	20 ± 2	13 ± 1	5

[a]5 mM Tris, 145 mM NaCl, 0.1% PEG, pH 7.4, 37 °C. Values for the slow form are in the presence of 145 mM choline chloride. Values for the fast form refer to the extrapolation $[Na^+] \rightarrow \infty$ at constant ionic strength of 145 mM. [b]Specificity ratio between the fast and slow forms. [c]In the presence of 5 mM $CaCl_2$ and 100 nM human thrombomodulin. [d]In the presence of 0.5 USP/mL of heparin.

in a constant saturation of the Na^+ site during all steps of thrombin catalysis or ligand recognition contradicts elementary principles of linkage thermodynamics and is fundamentally wrong. Na^+ binding and dissociation are the key molecular events that control substrate recognition by thrombin.

The binding of Na^+ to thrombin elicits significant spectral changes that can be followed by circular dichroism [61] or intrinsic fluorescence [7]. Earlier studies have documented significant changes in circular dichroism [75] and NMR [76] when Na^+ was exchanged with other monovalent cations, but the results were interpreted in terms of differential hydration phenomena. Orthner and Kosow [77] documented a large UV change upon replacing choline chloride with NaCl in the buffer solution and suggested that the change could be linked to a specific interaction of Na^+ with the enzyme altering the environment of a Tyr residue. In retrospect, the effect they measured probably originated from perturbation of the environment of Y225. The circular dichroism change indicates a slight increase in β structure upon Na^+ dissociation, which is difficult to pinpoint structurally. The intrinsic fluorescence increase upon Na^+ binding is compatible with burial of a Trp residue, which remains unidentified. The change in fluorescence is retained after deletion of W148 in the autolysis loop [44].

The binding constant for Na^+ and other monovalent cations can be measured accurately by fluorescence titration [7, 44, 61]. Na^+ is bound with an affinity about one order of magnitude higher than K^+, Li^+, Rb^+ and Cs^+. The origin of the higher specificity of Na^+ is due to steric factors in the cavity hosting the metal ion. Valence calculations show that the density occupied by Na^+ in the crystal structure has a high Na^+-specific valence and a low valence specific for Li^+ or K^+ [78]. K^+ is too big to fit the cavity without disturbing the network of water molecules. Rb^+ has an ionic radius similar to K^+ and binds with a slightly different coordination geometry compared to Na^+. Water 416 (fig. 3) is released in the presence of Rb^+ and in its place the carbonyl O atom of Y184a is recruited into the coordination shell [67]. This is due to the larger size of the cation, which makes it difficult to keep water 416 in the cavity bridging the Na^+ to the carbonyl O atom of Y184a. Apparently this change in coordination weakens the binding affinity. Li^+, on the other hand, appears to be too small to guarantee optimal bond strength with the surrounding water molecules. The rigid backbone architecture in the loop cannot tighten up the cavity, thereby leaving a loose bond environment around the small monovalent cation. Structural considerations, together with energetic calculations based on the valence, indicate that Na^+ has the optimal requirements of ionic radius and charge density to be accommodated in the cavity.

The energetic signatures of Na^+ binding are noteworthy [79]. The interaction of this cation with thrombin is linked to a large and negative heat capacity change of -1.1 ± 0.2 kcal/mol/K. The modest free energy change linked to Na^+ binding is the result of two large enthalpic and entropic contributions that compensate each other. A consequence of the large enthalpy change is that the equilibrium dissociation constant, K_d, increases from about 25 mM at 25 °C to about 110 mM at 37 °C. Hence, under physiological concentrations of NaCl (145 mM) the fast form is populated by only 56% of the molecules at 37 °C, as opposed to nearly 85% at 25 °C. The properties of thrombin in vivo are the average of those of the slow and fast forms and are optimally poised for allosteric regulation, whereas those measured at room temperature predominantly reflect the contribution of the fast form.

A summary of the key interactions of thrombin under physiological conditions of salt, pH and temperature is given in table 2. It is evident that the properties observed in the presence of 145 mM NaCl lie between those of the slow and fast forms. The fast form has higher specificity

for fibrinogen, releasing fibrinopeptide A 23-fold faster than the slow form. A similar effect is seen for the release of fibrinopeptide B from fibrin I. The fast form also cleaves the thrombin receptor 1 with a significantly higher specificity (39-fold), binds thrombomodulin with an affinity 9-fold higher and is inhibited by antithrombin III in the presence of heparin at a five-fold faster rate. The only interaction that shows a small, but significant preference for the slow form is the cleavage of protein C in the presence of thrombomodulin. This preference is even more significant (seven-fold) in the absence of thrombomodulin [23].

The large temperature effect on the Na^+ affinity is contrasted by a modest effect of ionic strength. The value of $\Gamma_{salt} = -d\ln K_d/d\ln[salt]$ is -0.25 ± 0.05, implying that a change in K_d by a factor of ten requires a change in ionic strength by a factor of 10,000. Current theories on the origin of monovalent cation specificity in proteins emphasize the role of electrostatic forces [80, 81]. Weak electrostatic potentials from the protein preferentially dehydrate K^+ over Na^+ and account for the prevalence of K^+-activated enzymes (see last section). Specific binding of Na^+ over K^+ would require strong electrostatic potentials to overcome the higher hydration barrier of the smaller cation [82–84] and guarantee optimal dehydration and preferential interaction with the protein. In the case of a protein like thrombin, which binds Na^+ with higher specificity than K^+, these theories predict the existence of a strong electrostatic potential at the level of the Na^+ binding site an a significant effect of ionic strength on the binding interaction. Nonlinear Poisson-Boltzmann calculations indicate that the region surrounding the Na^+ binding site has a strong negative electrostatic potential reinforced by the uncompensated charges of the nearby side chains of D189 and E192. The potential, however, is only minimally affected by Na^+ binding, which accounts for the small ionic strength dependence of this interaction [85].

As for the ionic strength dependence of binding, the large and negative heat capacity change cannot be reconciled with current theories on the role of the hydrophobic effect in molecular recognition and protein folding [86, 87]. The value is comparable to that measured for the folding of proteins like parvalbumin and ribonuclease A, or the binding of the hirudin N-terminal fragment 1–49 to thrombin [88], where the process is linked to burial of extended hydrophobic surface or large folding transitions. For a change of -1.1 kcal/mol/K, the predicted nonpolar surface buried upon binding must be on the order of 2600 Å^2 [87]. This value is unrealistically high for the thrombin-Na^+ interaction, corresponding to nearly 35% of the entire accessible hydrophobic surface area of the enzyme [88]. Alternatively, a large heat capacity change can arise from coupled folding transitions [86]. A predicted 70 residues must order upon Na^+ binding. We must therefore conclude that the slow form of thrombin is more disordered than the fast form, and that Na^+ binding causes nearly 25% of the enzyme to fold. This scenario is unrealistic in view of the highly constrained environment of the Na^+ binding site [67–69] and is also inconsistent with circular dichroism studies which demonstrate that the fast form is actually slightly more disordered than the slow form [61, 67]. We speculate that the predominant origin of the large heat capacity change upon Na^+ binding to thrombin must be found in the conspicuous water channel that embeds the Na^+ environment (fig. 3). The hydrogen bonding network of the water molecules in this region is remarkably complex and bridges residues of the enzyme that could not interact otherwise. This network of buried water molecules may be affected significantly upon release of Na^+.

Solvation of charged and polar groups is a process accompanied by a negative heat capacity change, the magnitude of which is small and precisely known only for simple molecules [89]. The thermodynamic signatures of polar group solvation must depend on where these groups are located. Inside a protein, as is the case for the Na^+ binding site of thrombin, solvating water molecules experience a drastic entropy loss compared to bulk water. This is in contrast with water molecules solvating groups on the surface of a protein. Likewise, the heat capacity of water molecules sequestered in the interior of a protein must be significantly lower than that of water molecules in the bulk solvent, because of their reduced mobility and more ordered structure. Burial of water molecules linked to ligand binding or protein folding may then result in large and negative heat capacity changes and contribute significantly to the effects of thermal transitions observed experimentally, as first suggested by Sturtevant [90]. This possibility seems particularly appropriate in the case of Na^+ binding to thrombin, where the contribution from hydration and burial of water molecules is well documented by the crystal structure.

The conservation of clusters of buried water molecules is a structural motif present throughout the serine protease family. These clusters are frequently, shaped as water channels forming extensive hydrogen-bonding networks linked to the protein backbone. In subtilisin, the water channel plays a structural role [91]. The locations of buried water molecules in the trypsin-like proteases are more extensive. Bartunik et al. [47] have identified a water channel in trypsin extending from the specificity pocket to the surface of the enzyme. Sreenivasan and Axelsen [92] have located 16 internal water sites conserved in trypsin, trypsinogen, chymotrypsin, chymotrypsinogen, elastase, kallikrein, tonin, and mast cell protease. Five of these water sites correspond to regions homologous to the Na^+-binding loop in thrombin. Upon examination of the structure of trypsin, it was shown that most water molecules are located in large clusters [93]. The study additionally found that the positions of buried water molecules in various trypsin structures are highly conserved and well ordered, displaying high occupancy and

low mobility. These internal water molecules are predominantly hydrogen-bonded to backbone atoms of the enzyme.

The wealth of information on the water molecules occupying the specificity pocket of serine proteases can be exploited to understand the molecular mechanism of Na^+ binding to thrombin. The guiding hypothesis is that water molecules conserved among all proteases should serve structural purposes and contribute to the stability of the environment of the specificity pocket. Water molecules present exclusively in thrombin should reveal the changes necessary for Na^+ binding, and should provide clues on the molecular environment of the water channel in the slow form, the crystal structure of which remains elusive [68].

Of the 16 solvent molecules located in the primary specificity pocket of thrombin, eight are conserved with trypsin and factor Xa, five with elastase and chymotrypsin, four with leukocyte elastase, and three with tonin [69]. Figure 3 shows that the core of the hydrogen-bonding network in thrombin is dominated by linkages conserved with trypsin. Removal of Na^+ and its connected water molecules leaves a trypsin-like network behind. This residual network in thrombin depends almost exclusively on hydrogen bonds conserved with trypsin. The solvent molecules assigned to structural roles are 405w, 423w, 427w, 429w, 462w, and 494w. 427w shares a hydrogen bond with 447w. 405w hydrogen bonds with the side chain of D189, the carbonyl O atom of A183, the side chain of Y228, and the N atom of G226, linking three of the β strands delimiting the water channel. 429w stabilizes the Na^+-binding environment by bridging W215 and F227. 405w, 427w, 447w, 462w, and 494w hydrogen bond with the side chain of D189. Solvent molecules assigned to Na^+-coordinating roles are 410w, 416w, 419w, 430w, and 431w. 416w and 419w directly coordinate Na^+, whereas 410w, 430w, and 431w are connected to these water molecules by hydrogen bonding. The loss of Na^+ should obviate the need for these solvent molecules in the slow form. 445w and 447w are special in that they play both structural and Na^+-coordinating roles. 445w hydrogen bonds both to the Na^+-binding and structural domains of the water channel. Upon the conformational switch to the slow form, 445w may gain a hydrogen bond with 447w, stabilizing both water molecules and preventing them from being lost with the Na^+.

The participation of Na^+ in the water channel of the fast form of thrombin implies a necessary rearrangement of the hydrogen-bonding partners upon the conformational shift to the slow form. Upon the departure of Na^+, the solvent molecules must compensate for the loss of a key partner in the hydrogen-bonding network. While Na^+ is coordinated by R221a and K224 in thrombin, the water molecule in trypsin conserved with 447w is the primary recipient of hydrogen bonds from residues 221a and 224. This is facilitated by a $\sim 70°$ difference in the orientation of the carbonyl O atom of residue 224 between thrombin and trypsin induced by the presence of P225 [8]. Considering that 447w participates in more hydrogen bonds than any other water molecules in thrombin, it is likely that the network is rerouted to 447w upon the departure of Na^+ in order to minimize perturbations. The water channel in the specificity pocket cannot leave R221a and K224 without hydrogen-bonding recipients, a situation not observed in any protease [69], and the absolutely conserved 427w

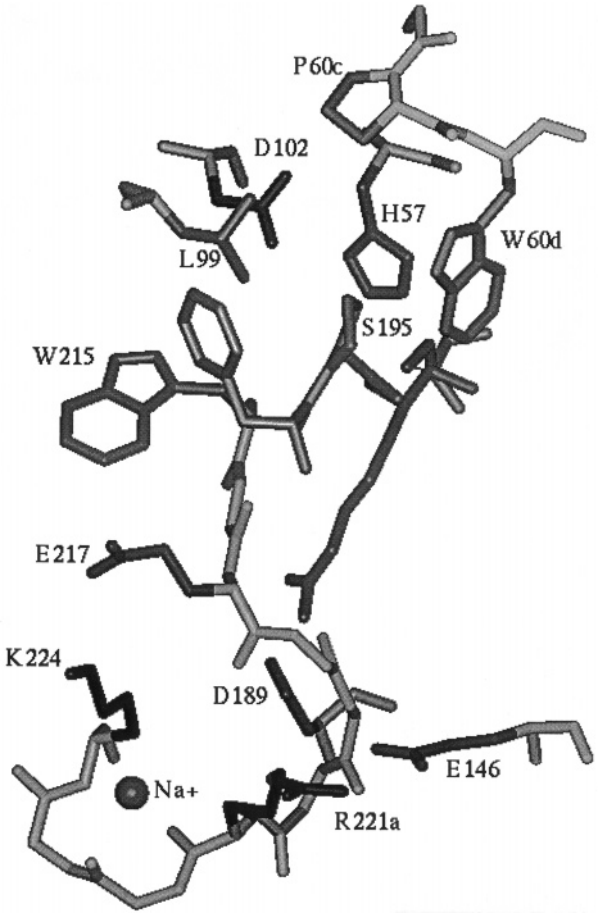

Figure 4. Contacts between the irreversible inhibitor H-D-Phe-Pro-Arg-CH_2Cl and the active site of thrombin. Shown are the thrombin residues D189, P60c, W60d, L99 and W215 that interact with the inhibitor. The guanidyl group of the Arg at P1 makes an ion pair with the carboxyl group of D189 at S1 at the bottom of the active site. Pro at P2 packs optimally in the S2 apolar cavity provided by the W60d loop. H-D-Phe at P3 makes favorable hydrophobic contacts in the cleft with L99 and especially a perpendicular aryl-aryl edge-on interaction with W215 at S3. The D enantiomer of Phe at P3 makes it possible to interact favorably with the aromatic moiety at S3 with minimum strain in the backbone of the inhibitor, fixed by Pro at P2. Also shown are the residues of the catalytic triad and the backbone of the 215–224 segment comprising the Na^+ binding loop. R221a is ion-paired to E146 in the neighbor autolysis loop, whereas K224 is ion-paired to E217.

Table 3. Substrate library for the analysis of thrombin specificity.

Abbreviation	Substrate	Site(s) perturbed
FPR	H-D-Phe-Pro-Arg-*p*-nitroanilide	none
FPK	H-D-Phe-Pro-Lys-*p*-nitroanilide	P1
FGR	H-D-Phe-Gly-Arg-*p*-nitroanilide	P2
VPR	H-D-Val-Pro-Arg-*p*-nitroanilide	P3
FGK	H-D-Phe-Gly-Lys-*p*-nitroanilide	P1 and P2
VPK	H-D-Val-Pro-Lys-*p*-nitroanilide	P1 and P3
VGR	H-D-Val-Gly-Arg-*p*-nitroanilide	P2 and P3
VGK	H-D-Val-Gly-Lys-*p*-nitroanilide	P1, P2 and P3

would be destabilized due to the loss of hydrogen-bonding partners.

Na^+ binding in thrombin may be influenced by the presence of an insertion loop at the opening of the water channel. The fast form of thrombin differs from trypsin and factor Xa in the size of the opening of the water channel on the enzyme surface. The 186a–d insertion loop in thrombin is preceded by P186, which shifts the rim of opening downward and increases the aperture of the channel. As a result, there is sufficient room to accommodate 416w and 419w. In trypsin, these two key water molecules would be uncomfortably close to the backbone of the 184–188 loop. The alternate positioning of residues 184 and 185 in thrombin due to the 186a insertion prevents them from participating in Na^+ coordination as in factor Xa [68]. As a result, water molecules 416w and 419w are employed to replace the two missing hydrogen-bonding partners. These two water molecules are in turn anchored to the 184–188 loop both directly and indirectly through additional water molecules that may be lost along with Na^+. The effect of this insertion may be to permit the departure of Na^+ from the specificity pocket with only a few solvent molecules. The insertion loop 186a–d must therefore influence the kinetics of Na^+ binding and dissociation, and possibly the large heat capacity change linked to Na^+ binding. In the slow form, the 184–188 loop may shift upward to fill the gap formerly occupied by Na^+ and the water network originating from the secondary elements 416w and 419w, giving the specificity pocket a trypsin-like conformation. 445w may shift upward by as much as 1 Å to accommodate the shift of the 184–188 loop and form a hydrogen bond with 447w.

The hypothesized changes in the specificity pocket that would occur during the allosteric transition of thrombin to the slow form can be summarized as follows. 447w becomes the primary element of the hydrogen-bonding network of the water channel as R221a and K224 reorient their carbonyl O atoms to this water molecule. The Na^+-binding portion of the network including 410w, 416w, 419w, 430w, and 431w is lost and 445w and 447w assume an exclusively structural role. The hydrogen-bonding connection between the bound Na^+ and the side chain of D189, passing through 427w in the fast form, is rerouted through 423w. Consequently, 427w may lose its contact with the $O^{\delta 2}$ atom, causing the side chain of D189 to reorient slightly. This change may be sufficient to alter the catalytic register for bound substrates with Arg at P1 making an ion pair with D189 and cause the increase in K_m and decrease in k_{cat} observed experimentally (see sections below). The 184–188 loop moves closer to the Na^+-binding loop, occupying the gap left by Na^+ and its accompanying solvent cage, which narrows the entrance to the channel. Squeezing some of the water molecules out of the channel may also reduce the accessibility of the specificity pocket and decrease the rate of diffusion of the substrate into the primary specificity site contributing to the reduced specificity of the slow form.

The interaction with chromogenic substrates

The crystal structure of thrombin inhibited with H-D-Phe-Pro-Arg-CH_2Cl [24] provides information on the interactions of the P1–P3 groups of the substrate with the enzyme. Arg at P1 makes an ion pair with D189 at S1 at the bottom of the catalytic pocket, Pro at P2 interacts with the S2 site formed by P60b, P60c and W60d, whereas H-D-Phe at P3 forms a favorable edge-to-face interaction with the aromatic ring of W215 at S3 (fig. 4). The D enantiomer at P3 is necessary to mimic the interaction of F8 of fibrinogen (table 1) with W215 of thrombin after the β-turn of the Aα chain following the Gly-Gly-Gly P3–P5 flexible region made to re-enter the catalytic pocket in a parallel configuration to the β-strand hosting W215 [94–96]. These interactions were mimicked with the chromogenic substrate FPR (table 3). The 30-fold difference in specificity for FPR between the slow and fast forms (table 4) echoes the effect seen for fibrinogen (table 2) and reinforces the relevance of the comparison. Furthermore, FPR provides a prototype for the interaction of many active-site inhibitors with the enzyme.

Site-specific thermodynamics [97, 98] offers a novel strategy to unravel the energetics of substrate recognition from the properties of a library containing all possible sequences with P sites carrying one of two possible residues. The basic advantage of this approach is that it involves a limited number of substrates and casts the energetics of recognition in terms of the same formalism as cooperative ligand binding. The S1–S3 sites of thrombin were probed by perturbations introduced in the P1–P3 sites of FPR, chosen to reduce the interactions of this substrate without causing unfavorable steric contacts, electrostatic and hydrophobic interactions. This ensured a gradual loss of specificity necessary for accurate experimental determination of the catalytic parameters for all members of the library.

Table 4. Specificity constants k_{cat}/K_m (in $\mu M^{-1}s^{-1}$) for the hydrolysis of synthetic substrates by wild-type and mutant thrombins[a].

	FPR	FPK	FGR	VPR	FGK	VPK	VGR	VGK
Fast form								
wt	90	7.9	2.0	100	0.021	2.1	0.34	0.0047
R221aA	80	4.6	0.75	36	0.011	0.96	0.14	0.0024
K224A	44	7.7	0.93	24	0.027	1.4	0.17	0.0044
R221aA/K224A	26	3.2	0.33	13	0.011	0.70	0.049	0.0017
Slow form								
wt	3.0	0.35	0.86	6.7	0.0026	0.11	0.17	0.00079
R221aA	1.6	0.040	0.042	1.0	0.00038	0.0097	0.0086	0.00013
K224A	0.47	0.034	0.012	0.28	0.00039	0.0063	0.0020	0.00013
R221aA/K224A	0.34	0.010	0.0025	0.077	0.00021	0.0018	0.00063	0.000063

[a]Experimental conditions: 5 mM Tris, I = 200 mM, 0.1% PEG, pH 8.0 at 25 °C. The slow form was studied in the presence of 200 mM choline chloride. The properties of the fast form refer to the limit $[Na^+] \to \infty$, at constant I = 200 mM. Errors are typically $\pm 2\%$.

H-D-Phe was replaced with H-D-Val in VPR, VPK, VGR and VGK, to replace the aromatic moiety with a hydrophobe. Pro was replaced with Gly in FGR, FGK, VGR and VGK, to avoid unfavorable steric contacts with S2 and relieve the rigidity caused by Pro at P2. Arg was replaced with Lys in FPK, FGK, VPK and VGK, to preserve the positive charge at P1 needed to contact D189 at S1. The resulting library (table 3) contains the reference substrate FPR, the three singly-substituted substrates FPK, FGR and VPR, the three doubly-substituted substrates FGK, VPK and VGR and the triply-substituted substrate VGK that are the eight species required for a complete site-specific analysis of a system of three sites existing in two possible states [99].

Analysis of the specificity constants for the library (table 4) reveals the cost of a single-site substitution (ΔG_1, ΔG_2 and ΔG_3) reflecting the local environment of each specificity site. In addition, it provides information on interactions involving double (ΔG_{12}, ΔG_{13}, ΔG_{23}) and triple (ΔG_{123}) substitutions (table 5) that reflect more global conformational constraints imposed by the specificity sites of the enzyme on the bound substrate. Large and significant nonadditivity is seen in the perturbations of the P1–P3 sites, the extent of which changes for each pair of substitutions and is sensitive to the allosteric state of the enzyme. When a site is perturbed, perturbation at a second site reduces specificity beyond simple additivity of the single perturbations. The difference between the second-order and third-order coupling free energies, $\Delta G_{12} + \Delta G_{13} + \Delta G_{23} - \Delta G_{123}$, shows that the coupling between any two perturbations is enhanced by more than 1 kcal/mol when the third site is perturbed. The dependence of the coupling between two sites on the third site is a signature of concerted interactions among the sites [97, 98] and suggests that the P1–P3 sites function as an allosteric switch, the energetic state of which is influenced globally by the particular residue present at any of the three sites. This effect originates from constraints imposed by the enzyme and the extent of nonadditivity is a measure of the conformational strain experienced by the bound substrate in the transition state. Consistent with this hypothesis, no interactions are observed for trypsin [99], which is devoid of Na^+-induced allosteric regulation [8] and has a more accessible environment of the specificity sites [47] compared to thrombin [24].

A consequence of the peculiar nonadditivity is that the free energy cost of a replacement made at any of the P1–P3 sites depends on the residue present at other sites (table 6). The free energy change to replace Arg with Lys at P1 is positive in both the slow and fast forms, for wild-type and mutant thrombins, indicating a loss of specificity. A similar finding is observed for trypsin and is due to the change in interaction with D189 in the specificity pocket S1 [99]. The side chain of Arg at P1 is long enough for the guanidinium group to form an ion pair with D189 (fig. 4), but Lys at P1 interacts with D189 through a water-mediated contact [100]. The change in

Table 5. Free energy values (in kcal/mol) due to perturbation of the P1–P3 sites[a].

	ΔG_1	ΔG_2	ΔG_3	ΔG_{12}	ΔG_{13}	ΔG_{23}	ΔG_{123}
Fast form							
wt	1.4	2.3	−0.1	1.3	0.8	1.1	2.2
R221aA	1.7	2.8	0.5	0.8	0.5	0.5	1.2
K224A	1.0	2.3	0.4	1.1	0.7	0.6	1.8
R221aA/K224A	1.2	2.6	0.4	0.8	0.5	0.7	1.5
Slow form							
wt	1.3	0.7	−0.5	2.2	1.2	1.4	3.3
R221aA	2.2	2.2	0.3	0.6	0.6	0.7	1.0
K224A	1.6	2.2	0.3	0.5	0.7	0.8	0.8
R221aA/K224A	2.1	2.9	0.9	−0.6	0.1	−0.1	−0.8

[a]Values were obtained from the specificity constants $s = k_{cat}/K_m$ in table 4 as follows (the suffix refers to the sequence of the substrate): $\Delta G_1 = -RT\ln(s_{FPK}/s_{FPR})$, $\Delta G_2 = -RT\ln(s_{FGR}/s_{FPR})$, $\Delta G_3 = -RT\ln(s_{VPR}/s_{FPR})$, $\Delta G_{12} = -RT\ln(s_{FGK}s_{FPR}/s_{FPK}s_{FGR})$, $\Delta G_{13} = -RT\ln(s_{VPK}s_{FPR}/s_{FPK}s_{VPR})$, $\Delta G_{23} = -RT\ln(s_{VGR}s_{FPR}/s_{FGR}s_{VPR})$, $\Delta G_{123} = -RT\ln(s_{VGK}s^2_{FPR}/s_{FPK}s_{FGR}s_{VPR})$. Errors are typically ± 0.1 kcal/mol.

Table 6. Free energy change (in kcal/mol) in specificity due to perturbation of the P1–P3 sites[a].

	Fast form				Slow form				Coupling			
	wt	R221aA	K224A	R221aA/ K224A	wt	R221aA	K224A	R221aA/ K224A	wt	R221aA	K224A	R221aA/ K224A
Replacement at P1 (Arg → Lys)												
FPX	1.4	1.7	1.0	1.2	1.3	2.2	1.6	2.1	0.2	−0.5	−0.5	**−0.8**
FGX	2.7	2.5	2.1	2.0	3.4	2.8	2.0	1.5	**−0.7**	−0.3	0.1	0.5
VPX	2.3	2.1	1.7	1.7	2.4	2.7	2.2	2.2	−0.1	−0.6	−0.6	−0.5
VGX	2.5	2.4	2.2	2.0	3.2	2.5	1.6	1.4	−0.6	−0.1	0.5	0.6
Replacement at P2 (Pro → Gly)												
FXR	2.3	2.8	2.3	2.6	0.7	2.2	2.2	2.9	**1.5**	0.6	0.1	−0.3
FXK	3.5	3.6	3.3	3.4	2.9	2.8	2.6	2.3	0.6	**0.8**	**0.7**	**1.1**
VXR	3.4	3.3	2.9	3.3	2.2	2.8	2.9	2.8	**1.2**	0.5	0.0	0.5
VXK	3.6	3.5	3.4	3.6	2.9	2.6	2.3	2.0	**0.7**	**1.0**	**1.1**	**1.6**
Replacement at P3 (Phe → Val)												
XPR	−0.1	0.5	0.4	0.4	−0.5	0.3	0.3	0.9	0.4	0.2	0.1	−0.5
XPK	0.8	0.9	1.0	0.9	0.7	0.8	1.0	1.0	0.1	0.1	0.0	−0.1
XGR	1.0	1.0	1.0	1.1	1.0	0.9	1.1	0.8	0.1	0.1	−0.1	0.3
XGK	0.9	0.9	1.1	1.1	0.7	0.6	0.7	0.7	0.2	0.3	0.4	0.4

[a]Listed are all possible configurations of the other two P sites in the corresponding substrate. Errors are ± 0.1 kcal/mol or less. Values were obtained from the data in tables 4 and 5. The difference between the values for the fast and slow forms gives the coupling free energy between the substitution and the slow → fast transition [98]. Positive values are indicative of stabilization of the slow form in the transition state, whereas negative values signal stabilization of the fast form. Values of the coupling in excess of $\pm RT$ (0.6 kcal/mol) are in bold.

thrombin varies from 1.4 kcal/mol when Pro is at P2 to about 3 kcal/mol when Gly is at P2. The environment of D189 in thrombin is therefore less forgiving when the substrate acquires more flexibility with the Pro → Gly replacement at P2. The free energy change due to Phe → Val replacement at P3 shows that VPR is a slightly better substrate for thrombin than FPR itself, contradicting the unique virtues of an aromatic residue at P3 documented by the crystal structure [24]. A loss of specificity is however observed with Val at P3 when a replacement is made at P1 or P2, again as a result of the interactions among the P1–P3 sites. Minimal changes in specificity are seen for trypsin and plasmin, indicating a lack of interaction of Phe or Val with the S3 site in these proteases [99].

The energetic difference between the fast and slow forms (table 6) is a measure of the coupling between the substitution at a given P site and the slow → fast conversion in the transition state. A positive value of this difference indicates that the replacement opposes Na^+ binding and stabilizes the slow form, or that the replaced residue binds preferentially to the fast form. A negative value indicates an enhancement of Na^+ binding and stabilization of the fast form, or that the replaced residue binds preferentially to the slow form [98, 99]. The Arg → Lys replacement at P1 slightly promotes the slow → fast transition when Gly is present at P2. On the other hand, the Pro → Gly replacement at P2 strongly stabilizes the slow form. The replacement at P3 is inconsequential on the allosteric equilibrium. This provides information on the molecular events linked to the allosteric transition of thrombin from an anticoagulant to a procoagulant enzyme. The transition mostly affects the environment of the S2 site, with modest effects on the S1 site and none on the S3 site. Constraints at the S2 site accounts for the lower specificity of the slow form compared to the fast form and become inconsequential if the substrate acquires flexibility with a Gly at P2 and can readjust in the active site to compensate for the increased steric hindrance of the S2 site in the slow form. These findings explain why the thrombin mutant W60dS cleaves FPR with the same specificity in the slow and fast forms [38] and suggest the bulky side chain of W60d as the probable origin of the constraints at S2.

The molecular origin of the peculiar nonadditivity seen for thrombin depends entirely on the strength of the R221a-E146 and K224-E217 ion pairs. Disruption of the R221a-E146 ion pair affects S1 and S2 mainly in the slow form and reduces the extent of coupling among substitutions at the P1–P3 sites. This may be due to enhanced mobility of the autolysis loop on the Glu side of the ion pair upon disruption of the contact. In addition, the R221aA replacement may affect the orientation of the carbonyl O atom and cause a rearrangement of the water molecules in the channel embedding the S1 site [69]. This ion pair is energetically stronger in the slow form, where it may increase the rigidity of the neighbor autolysis loop and trigger the release of Na^+

Table 7. Thermodynamic parameters for the interaction of hirudin and its fragments with thrombin[a].

	K_d	ΔG (kcal/mol)	ΔC_P (kcal/mol/K)	Γ_{salt}	K_d	ΔG (kcal/mol)	ΔC_P (kcal/mol/K)	Γ_{salt}
	Fast form				Slow form			
hir	0.16 ± 0.02 pM	-17.45 ± 0.07	-1.7 ± 0.2	-1.22 ± 0.09	4.4 ± 0.4 pM	-15.49 ± 0.05	-1.6 ± 0.2	-1.89 ± 0.07
hir^{1-49}	52 ± 3 nM	-9.94 ± 0.03	-1.2 ± 0.1	-0.16 ± 0.06	1.3 ± 0.1 μM	-8.03 ± 0.05	-1.1 ± 0.1	-0.21 ± 0.09
hir^{1-43}	0.25 ± 0.02 μM	-9.01 ± 0.05	-0.9 ± 0.1	n.d.	2.5 ± 0.2 μM	-7.64 ± 0.05	-0.8 ± 0.2	n.d.
hir^{55-65}	2.3 ± 0.3 μM	-7.69 ± 0.08	-0.7 ± 0.2	-1.06 ± 0.02	14.7 ± 0.8 μM	-6.59 ± 0.03	-0.7 ± 0.2	-1.35 ± 0.09

[a]Values of K_d, ΔG and Γ_{salt} refer to 25 °C. hir, hirudin; hir^{1-49}, fragment 1–49; hir^{1-43}, fragment 1–43; hir^{55-65}, fragment 55–65. n.d., not determined.

and some of its ligating water molecules. Disruption of the K224–E217 ion pair produces effects very similar to those seen for the R221aA mutant, with a reduction of the coupling among the P1–P3 sites especially in the slow form. This ion pair bridges two residues on the last two β-strands of the B chain and contributes to the integrity of S1 and S2 in the slow form. The region in immediate proximity to K224 and E217 plays a key role in substrate selectivity and is absolutely conserved in thrombin from different species [70]. The state of this ion pair probably influences the optimal orientation of the substrate for binding to S1 and S2. The results on the double mutant point out a weak synergism between the two ion pairs in the slow form. Interactions among the P1–P3 sites are practically abolished in this mutant and its energetic profile resembles that of trypsin and plasmin which lack the two ion pairs [99].

The presence of strong negative coupling among the P1–P3 sites represents an important complication in the optimization of active site inhibitors. In fact, a lack of optimal interaction at any one site will negatively influence specificity at all three sites. Optimal binding to the active site of thrombin demands a rigid backbone around the P2–P3 position, coupled with a strong interaction with S1 and a hydrophobic group at P3. Pro or pipecolyl derivatives at P2 should ensure rigidity of the P2–P3 bond. Arg or equally bulky, positively charged groups like guanidinophenylalanine derivatives should provide strong electrostatic coupling with S1. Val, Leu, Ile or highly hydrophobic unnatural amino acid should be used at P3. The importance of a strong coupling with S1 has long been recognized [101], although specific binding can be achieved with other groups at P1 [102]. Other essential factors are the rigidity of the P2–P3 bond coupled to the hydrophobicity of P3. Introduction of a hydrophobic group at P3 may bring about a more favorable interaction with the moiety of L99 close to the apolar site S2. In fact, residue Y3 of hirudin contacts W215 of thrombin in an edge-to-face interaction [54], but replacement of Y3 with hydrophobic residues results in enhanced binding affinity [103].

The interaction with hirudin

Among the natural inhibitors of thrombin, hirudin is by far the most potent and selective. This protein is derived from the salivary glands of the European leech *Hirudo medicinalis* [104] and binds to thrombin with a dissociation constant in the fM range [105]. The importance of hirudin for studies of thrombin structure-function correlations stems from the large number of contacts made with the enzyme and its extraordinary specificity. Hirudin is in fact an excellent probe of the allosteric state of thrombin because it covers about 12% of the available surface area of the enzyme upon formation of the complex [54]. Hirudin binding also serves as a paradigm

for other thrombin interactions of physiological interest, like those involving fibrinogen and the thrombin receptors. The C-terminal domain of hirudin, on the other hand, mimics most of the effects of thrombomodulin.

Crystallographic analysis of the thrombin-hirudin complex has documented a wealth of polar and hydrophobic interfacial contacts [53,54], which account for the high specificity of the inhibitor. Stone has studied the effect of amino acid substitutions extensively. He has found that replacement of the acidic residues in the C-terminal domain of the inhibitor leads to reduced association rates and affinity [106, 107]. Replacement of hydrophobic residues also lowers the affinity [108]. Finally, significant contributions to the binding free energy come from nonpolar residues in the compact N-terminal domain of the inhibitor [109, 110]. These findings have led to the formulation of a plausible mechanism for hirudin binding in terms of an electrostatic steering effect, originating from the coupling between the positively charged exosite I and the negatively charged C-terminal domain of the inhibitor [111]. More recent calculations account for the hydration effects and predict the correct energetics of binding for several mutants of the acidic C-terminal domain [112]. Once the complex is formed, a conformational change optimizes the docking at the active site of the hydrophobic N-terminal domain [60]. This mechanism accounts for the significant ionic strength dependence of the binding of hirudin and its C-terminal fragment to thrombin (table 7).

Hirudin discriminates quite well between thrombin forms. It binds to the fast form with an equilibrium dissociation constant in the fM range, which is increased about 30-fold when the enzyme switches to the slow form [61]. Comparison of the NMR structure of free hirudin [113, 114] and the crystal structure of the thrombin-hirudin complex [54] reveals that the C-terminal fragment 50–65 of the inhibitor undergoes a large conformational transition upon binding, from a disordered to a folded 3_{10}-helix conformation. This gives rise to a large heat capacity change upon binding (table 7) in either the slow or fast form [88]. An unusually large negative change in heat capacity, and an entropy deficit linked to formation of a protein-ligand complex, suggests an induced-fit mechanism of recognition arising from a folding event [86]. This mechanism implies that a conformational change follows the binding interaction to promote or enhance complementarity between the protein and the ligand, consistent with kinetic data [60]. The conformational change appears in the observed thermodynamics as a larger than expected heat capacity change because folding the loop buries nonpolar surface beyond that observed to be buried in the interface. However, the favorable entropy from removing additional nonpolar surface area from water is offset by the entropic cost of constraining the conformation of amino acids in the loop. This effect from folding results in a smaller than expected entropy change. In contrast, rigid body interactions typically do not exhibit large negative heat capacity changes and do not have entropic costs associated with reducing conformational degrees of freedom. Many protease-inhibitor interactions appear to fall into this category. The interaction of trypsin with the soybean inhibitor, or subtilisin and chymotrypsin with the subtilisin inhibitor, are characterized by heat capacity changes in the range of -0.2 to -0.4 kcal/mol/K [115–117]. The case of thrombin-hirudin interaction is unusual: the heat capacity change is not only more pronounced than that observed for other protease-inhibitor interactions, but it is also more negative than that reported for many other protein-ligand interactions linked to large conformational changes [86]. The value of -1.7 ± 0.2 kcal/mol/K measured for thrombin-hirudin interaction is the largest heat capacity change ever reported for a protease-inhibitor interaction. Unlike other protease-inhibitor interactions, the thrombin-hirudin interaction is an induced-fit mechanism of recognition rather than a rigid-body association.

The origin of the large heat capacity observed for the thrombin-hirudin interaction is revealed by studies of hirudin fragments hir^{1-49} and hir^{1-43} [88]. Digestion by chymotrypsin removes the residues 50–65 which are disordered in the free protein in solution [113, 114], but order upon binding [54]. Digestion by endoprotease V8 removes six additional residues, yielding hir^{1-43}. These residues contribute six of 22 intramolecular hydrogen bonding interactions in the N-terminal compact core of hirudin. In particular, removal of K47 deletes contacts to T4, D5 and N12 and deletion of Q49 removes a hydrogen bond to K60f in the W60d loop. The absence of the C-terminal fragment weakens binding significantly, with K_d increasing five orders of magnitude. However, hir^{1-49} still discriminates between the two forms of thrombin and binds the fast form one to two orders of magnitude more tightly than the slow form (table 7). Deletion of six additional C-terminal residues to yield hir^{1-43} further reduces the binding affinity for thrombin in the fast form, but does not significantly affect binding to the slow form. A similar loss of specificity is observed for the C-terminal fragment hir^{55-65}. The heat capacity change decreases progressively as portions of hirudin are deleted. As for intact hirudin, the values for the fragments are independent of the allosteric state of thrombin, implying an origin in the inhibitor or similar structural changes in the complex in both forms. Surface area calculations fail to predict the whole extent of heat capacity change for hirudin and its fragments [88]. The assumptions made about regions of thrombin that fold upon binding of the inhibitor are inconsistent with structural data that demonstrate ordered structure in the enzyme without inhibitor [55]. The origin of the large heat capacity change upon binding of hirudin remains mostly elusive. As for Na^+ binding, a key role in the interaction can be played by water molecules, buried in the interior of the protein,

Table 8. Thermodynamic and kinetic parameters for the interaction of fibrinogen and fibrin I with thrombin[a].

	k_1 ($\mu M^{-1}s^{-1}$)	E_1 (kcal/mol)	k_2/k_{-1}	Γ_{salt}	k_1 ($\mu M^{-1}s^{-1}$)	E_1 (kcal/mol)	k_2/k_{-1}	Γ_{salt}
	Fast form				Slow form			
Fibrinogen	19 ± 4	12 ± 2	3 ± 2	-1.58 ± 0.07	2.5 ± 0.3	10 ± 1	4 ± 1	-1.50 ± 0.08
Fibrin I	9.1 ± 0.7	6.8 ± 0.9	11 ± 4	-2.43 ± 0.09	2.5 ± 0.2	2.1 ± 0.9	7 ± 2	-2.35 ± 0.07

[a]Values of k_1 (second-order rate for formation of the enzyme-substrate complex), k_2/k_{-1} (stickiness of the substrate, expressed as the ratio between the rates of acylation and dissociation of the complex) and Γ_{salt} refer to 25 °C. E_1 is the activation energy associated with k_1.

that rearrange upon binding. Other water molecules participate in the thrombin-hirudin interface [54]. Further investigation is needed to elucidate the role of these buried water molecules in the energetics of thrombin interactions.

A consequence of the similarity in heat capacity changes observed in the slow and fast forms of thrombin, upon binding of hirudin, is that the difference in binding affinity between the fast and slow forms increases with temperature, reaching almost 3 kcal/mol in the physiological temperature range [88]. The coupling free energy measured as the difference between the free energies of binding to the fast and slow forms [61] increases linearly (in absolute value) with temperature and the coupling enthalpy is constant and endothermic. Since the coupling free energy is negative over the entire temperature range, the driving force for preferential binding of hirudin to the fast form must be entropic. Preferential interaction with the fast form is due to the balance of two opposite forces, both quite large in magnitude. The contribution of enthalpic effects opposes the slow → fast transition and stabilizes binding to the slow form. The contribution of entropic effects favors the slow → fast transition and stabilizes binding to the fast form. In the physiological temperature range the entropic effects prevail and result in preferential binding of hirudin to the fast form.

The coupling free energy for hirudin and hir^{1-49} is practically identical throughout the temperature range from 5 to 45 °C, notwithstanding the difference in affinity of five orders of magnitude between the intact inhibitor and this fragment (table 7). This suggests that the thrombin residues responsible for recognition of hir^{1-49} store most of the regulatory free energy for switching from the slow to the fast form. One of these residues can be identified directly from comparison of the binding properties of hir^{1-49} and hir^{1-43}. These fragments bind to the slow form with the same affinity, whereas hir^{1-49} binds to the fast form with higher affinity compared to hir^{1-43}. In the case of hir^{1-43} the coupling free energy is less pronounced. This large effect compared to hir^{1-49} is due to removal of only six residues, one of which forms a hydrogen bond with K60f of thrombin. The side chain of K60f probably makes no contacts with hirudin in the slow form, since the affinity of hir^{1-43} or hir^{1-49} for this form is identical. The contribution of K60f to the coupling free energy is about -0.6 kcal/mol. The remaining -1.3 kcal/mol of preferential binding of the hir^{1-43} to the fast form at 25 °C may originate from polar contacts made by the fragment 1–43 of hirudin with thrombin. There are nine such contacts, seven hydrogen bonds and two ion pairs [54]. Six of these hydrogen bonds involve S214, G216, E217, G219 and K224 of thrombin. S214, G216 and E217 are on the penultimate β-strand of the B chain, in close proximity to the specificity site S3 of thrombin around W215 [24]. G219 and K224 are in the Na^+ binding loop. One of two ion pairs involves R221a, which is another residue of this loop. Contacts made by hirudin residues with R221a and K224, or with residues in close connection to the Na^+ binding loop (e.g., G216, E217), may be responsible for the preferential binding of hir^{1-43} to the fast form.

Recent mutagenesis studies of thrombin have identified an *allosteric core* of residues which are energetically linked to the slow → fast transition and determine the specificity for either allosteric form [37]. The allosteric core involves residues of the Na^+ binding loop, the W60d loop and the environment of E192 and E39, but not residues of exosite I and II. When a ligand targets residues in the allosteric core it stabilizes either the slow or fast form. Ligands that bind to residues outside the allosteric core experience only a small energetic difference between the two forms. Most of the contacts made by the N-terminal portion of hirudin are with the allosteric core, which explains why the 1–43 and 1–49 fragments of hirudin discriminate between the two thrombin forms better than the 55–65 C-terminal fragment.

The interaction with fibrinogen

Fibrinogen is the most important substrate of thrombin and circulates in the plasma as a dimer of three chains $(A\alpha B\beta\gamma)_2$, covalently linked by disulfide bonds [118, 119]. This molecule is converted into the insoluble fibrin clot by a series of enzymatic and physical steps. Important aspects of the physical steps of fibrin polymerization have been elucidated over the past fifty years [120–127]. Understanding the enzymatic steps has been greatly facilitated by HPLC analysis of the release of fibrinopep-

Table 9. Thermodynamic parameters for the interaction of thrombomodulin and its fragments with thrombin[a].

	K_d	ΔG (kcal/mol)	ΔC_P (kcal/mol/K)	Γ_{salt}	K_d	ΔG (kcal/mol)	ΔC_P (kcal/mol/K)	Γ_{salt}
	Fast form				Slow form			
hTM^+	3.4 ± 0.1 nM	-11.55 ± 0.02	-0.2 ± 0.1	-5.2 ± 0.3	18.8 ± 0.8 nM	-10.53 ± 0.02	-0.5 ± 0.2	-4.5 ± 0.4
hTM^-	26 ± 2 nM	-10.35 ± 0.05	-0.27 ± 0.07	-2.1 ± 0.3	180 ± 20 nM	-9.20 ± 0.07	-0.8 ± 0.1	-2.4 ± 0.1
rTM	1.8 ± 0.1 nM	-11.93 ± 0.03	-0.4 ± 0.1	-5.1 ± 0.4	16.8 ± 0.9 nM	-10.61 ± 0.03	-0.6 ± 0.2	-5.4 ± 0.1
TM^{4-5}	330 ± 20 nM	-8.84 ± 0.04	-0.7 ± 0.2	-2.1 ± 0.1	3.2 ± 0.2 μM	-7.50 ± 0.04	-0.99 ± 0.03	-2.3 ± 0.4
TM^{4-5-6}	3.4 ± 0.1 nM	-11.55 ± 0.02	-0.6 ± 0.1	-2.6 ± 0.1	22 ± 2 nM	-10.45 ± 0.05	-1.3 ± 0.3	-2.5 ± 0.2

[a]Values of K_d, ΔG and Γ_{salt} refer to 25 °C. hTM^+, human thrombomodulin with chondroitin sulfate; hTM^-, human thrombomodulin without chondroitin sulfate; rTM, rabbit thrombomodulin; TM^{4-5}, human thrombomodulin fragment containing EGF domains 4 and 5; TM^{4-5-6}, human thrombomodulin fragment containing EGF domains 4, 5 and 6.

tides [128–130], and by measurements of the equilibrium dissociation constant for thrombin-fibrinogen interaction [50]. Structural information is also available on the recognition of fibrinopeptide A by thrombin [94, 95]. Structural information on the fibrinogen molecule itself has recently been obtained [131].

The precise sequence of thrombin-catalysed events leading to clot formation has been elucidated by Shafer [129, 132, 133]. The Shafer mechanism states that fibrinogen cleavage by thrombin encompasses four steps. First, a highly-specific cleavage at the Aα chain leads to release of fibrinopeptide A and formation of fibrin I monomers. Second, these monomers aggregate to form fibrin I protofibrils. Third, a second highly-specific cleavage by thrombin at the Bβ chain of the fibrin I monomers leads to release of fibrinopeptide B and formation of fibrin II protofibrils. Fourth, these protofibrils rapidly aggregate to form the scaffold of the fibrin clot. This mechanism has been tested and confirmed under a variety of solution conditions [127].

The crystal structure of thrombin complexed with fibrinopeptide A covalently attached to S195 via a chloromethyl ketone moiety [94, 95] has revealed some important interactions with the bound substrate. Arg at P1 makes the expected ion pair with D189 at the bottom of the catalytic pocket, and Val at P2 finds a favorable environment in the apolar S2 site. The Gly-Gly-Gly P3–P5 sequence then enables a β-turn that causes the fibrinopeptide to exit and re-enter the active site. In this turn, the carbonyl O of G12 makes a minor van der Waals contact with the Cγ of E217 of thrombin, E11 makes an ion pair with R173 of thrombin and F8 engages into an edge-to-face interaction with W215 of thrombin, before the N terminus of the fibrinopeptide leaves the thrombin surface and becomes totally exposed to the solvent. Very little is known about the primed sites for fibrinogen. Early studies by Scheraga and Henschen have pointed out that the sequence 36–54 is important for recognition of exosite I [122, 134]. This sequence contains the characteristic combination of acidic and nonpolar residues also found in the C-terminal domain of hirudin.

Recent findings from site-directed mutagenesis studies have cast serious doubts on the validity of some of the conclusions drawn from the crystal structure of thrombin complexed with fibrinopeptide A [94, 95]. The ion pair between R173 of thrombin and E11 of fibrinogen was deemed to be a major determinant of fibrinogen recognition [94]. However, when the ion pair is broken in the R173A mutant, no significant effect on the release of fibrinopeptides is observed [37, 135]. On the other hand, replacement of E217 with Ala reduces specificity toward fibrinogen 40-fold [74], an effect that can hardly be accounted for by the tenuous contact between the Cγ atom of this residue and the carbonyl O atom of G12 of fibrinogen documented in the crystal structure. A similar loss (40-fold) of fibrinogen binding is obtained with the Ala replacement of K224 and R221a [9], two residues that do not contact the Aα chain according to the crystal structure. The most striking example is provided by the deletion of the autolysis loop E146-K149e which reduces fibrinopeptide A release 240-fold [44], though none of its residues makes contact with the substrate in the crystal structure. These discrepancies draw attention to the contribution of the N-terminal domains of the Bβ and γ chains of fibrinogen, not documented in the crystal structure, in determining the correct recognition of this substrate leading to the release of fibrinopeptide A and clotting. Mutations in exosite I show that R73 and Y76, but not R75, are involved in fibrinogen binding [37, 39]. Mutation of K60f in the W60d loop also reduces fibrinogen binding [39], whereas mutation of W60d affects predominantly recognition of fibrin I and the release of fibrinopeptide B [37, 38].

The higher specificity of the fast form toward fibrinogen leading to the release of fibrinopeptide A is due to a higher k_{cat} and a lower K_m [127], as seen for small chromogenic substrates [7]. Diffusion of fibrinogen into the active site of thrombin is enhanced in the fast form (table 8). In both forms, formation of a productive complex between fibrinogen and thrombin requires a remarkably small activation energy, indicating that the process is diffusion-limited. Fibrinogen is a sticky sub-

strate for thrombin and once the Michaelis-Menten complex is formed, the tendency of the reaction is to proceed in the direction of the chemical conversion of the acyl enzyme. Once the complex has been formed, there is a very high energy barrier for dissociation into the parent species and the reaction proceeds rapidly toward the acylation step, followed by deacylation and dissociation of fibrinopeptide A and fibrin I. The combination of a diffusion-controlled encounter, and a rate constant for acylation comparable to that for dissociation into the parent species, contribute to the optimization of the kinetic mechanism of recognition and hydrolysis of this thrombin substrate. Fibrin I shows some noteworthy differences compared to fibrinogen. Diffusion into the active site of thrombin is enhanced in the fast form, although the value is slightly lower than that of fibrinogen. The activation energy for formation of the Michaelis-Menten complex is significantly smaller than that observed for fibrinogen, indicating that there is practically no barrier, other than diffusion, opposing the interaction of fibrin I with thrombin. The stickiness of fibrin I is considerably more pronounced, independent of the allosteric state of thrombin.

The origin of the low energy barrier for formation of the enzyme-substrate complex is in the electrostatic steering that may help orient the enzyme and the substrate for optimal formation of the complex. This parallels the thrombin-hirudin interaction and suggests that binding to exosite I would follow a general nonspecific mechanism through which negatively charged ligands are attracted to the positively charged surface of thrombin. The value of Γ_{salt} is about -1.6 for the interaction with fibrinogen leading to release of fibrinopeptide A and -2.5 for the interaction with fibrin I leading to release of fibrinopeptide B. The marked difference between the two fibrinopeptides indicate differences in ionic contributions to formation of the transition state for fibrinogen and fibrin I. These contributions may include specific ion binding interactions with thrombin and fibrinogen or fibrin I, as well as nonspecific electrostatic effects. No difference in Γ_{salt} is detected when Na^+ is replaced by the inert choline, or Cl^- by the inert F^- [127], which implies that an important driving force for the formation of the thrombin-fibrinogen and thrombin-fibrin I complexes comes from electrostatic coupling. Electrostatic effects are more pronounced for the release of fibrinopeptide B.

As for the thrombin-hirudin interaction, the origin of preferential interaction of fibrinogen and fibrin I with the fast form of thrombin is entropic. Enthalpic components stabilize binding of fibrinogen and fibrin I to the slow form in the transition state, whereas entropic components favor binding to the fast form. In the temperature range of physiological interest the entropic components predominate and give rise to preferential binding to the fast form. Hence, electrostatic steering accounts for the low energy barrier to the encounter and the diffusion-controlled nature of the interaction of fibrinogen with thrombin. Once the complex in the transition state is formed, the enzyme shifts to the fast form due to its higher specificity, this shift being driven by entropic factors.

Table 10. Specific salt dependence of K_d (nM) for the binding of thrombomodulin and its fragments to thrombin[a].

	ChCl	NaCl	NaF
hTM^+	18.8 ± 0.8	3.8 ± 0.1	0.35 ± 0.02
hTM^-	180 ± 20	30 ± 2	11.4 ± 0.4
rTM1	6.8 ± 0.9	2.1 ± 0.1	0.14 ± 0.02
TM^{4-5}	3200 ± 200	380 ± 20	150 ± 5
TM^{4-5-6}	22 ± 2	3.8 ± 0.1	1.42 ± 0.04

[a]Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0 at 25 °C, in the presence of 200 mM salt as indicated. ChCl, choline chloride; hTM^+, human thrombomodulin with chondroitin sulfate; hTM^-, human thrombomodulin without chondroitin sulfate; rTM, rabbit thrombomodulin; TM^{4-5}, human thrombomodulin fragment containing EGF domains 4 and 5; TM^{4-5-6}, human thrombomodulin fragment containing EGF domains 4, 5 and 6.

The interaction with thrombomodulin and protein C

Thrombomodulin is a cofactor, present on the surface of endothelial cells, that markedly increases the ability of thrombin to activate protein C while inhibiting in a competitive manner fibrinogen binding [16]. Human thrombomodulin contains 557 residues and consists of five distinct domains: a 226-residue N-terminal domain with partial similarity to lectin-like molecules [136], a 236-residue domain containing six epidermal growth factor-like (EGF-like) domains, a glycosylated Ser/Thr-rich domain of 34 amino acids, a transmembrane domain of 23 amino acids and a C-terminal cytosolic tail of 38 residues [137–139]. The EGF-like domains 5 and 6 are essential for thrombomodulin binding to thrombin, whereas the EGF-like domain 4 is required for cofactor activity [140–145]. Crystallographic and NMR studies indicate that the EGF-like domains 5 and 6 bind thrombin at exosite I [51, 52]. Some thrombomodulin molecules contain a chondroitin sulfate moiety linked to the Ser/Thr-rich domain that precedes the transmembrane domain [146]. The chondroitin sulfate is present in rabbit thrombomodulin [146], but the fraction of human thrombomodulin containing the glycosaminoglycan moieties is unknown [147]. The chondroitin sulfate moiety does not contribute to the cofactor activity, but it is important for the direct anticoagulant activity of thrombomodulin. Rabbit thrombomodulin devoid of chondroitin sulfate retains the ability to enhance protein C activation, but has a reduced capacity of inhibiting fibrinogen clotting and activation of factor V [148]. In addition to promoting direct anticoagulant activity, the chondroitin sulfate moiety modestly accelerates the rate

of inhibition of thrombin by antithrombin III, whereas in the presence of heparin it has the opposite effect, which suggests a possible competition with heparin binding at the highly basic exosite II [149]. Recent studies support this possibility [62, 65, 66, 150].

Detailed studies carried out on thrombomodulin and its fragments, following the same strategy used for the interaction of thrombin with hirudin [88], have revealed the mechanism of action of this cofactor [62]. Thrombomodulin binds to the fast form of thrombin with higher affinity (table 9), as do rabbit thrombomodulin and the two thrombomodulin fragments $TM^{4\text{-}5}$ and $TM^{4\text{-}5\text{-}6}$. The increased affinity observed in the presence of the chondroitin sulfate moiety explains the lower anticoagulant activity of hTM^- compared to hTM^+ [146, 148]. The fragment $TM^{4\text{-}5\text{-}6}$ and rTM bind with an affinity similar to hTM^+, whereas $TM^{4\text{-}5}$ lacking the EGF-like domain 6 has an affinity 100-fold lower underscoring the importance of the 6th EGF-like domain in thrombin recognition [141, 142]. Binding of hTM^+ to thrombin is characterized by a modest heat capacity change in the fast form and a more significant change in the slow form. These effects are also seen for rabbit thrombomodulin and are not altered significantly by deletion of the chondroitin sulfate moiety. The heat capacity change originates from binding of the EGF-like domains to exosite I. The values measured for thrombin interaction with the fragments $TM^{4\text{-}5}$ and $TM^{4\text{-}5\text{-}6}$ are larger than those pertaining to hTM or hTM^- and confirm the dominant role played by the EGF-like domains in determining the heat capacity change for recognition by thrombin. In all cases, binding to the slow form causes a significantly larger heat capacity change by 0.3–0.6 kcal/mol/K, signaling the presence of distinct mechanisms of recognition by the two forms of thrombin. The interaction of thrombomodulin with the fast form of thrombin is a simple rigid-body association and the heat capacity change can be predicted entirely from the surface area removed from the solvent upon formation of the complex. This implies that the fast form is the optimal conformation for recognizing thrombomodulin, which must be a very rigid molecule in solution and does not change its conformation upon binding to the enzyme. On the other hand, binding to the slow form brings about additional contributions to the heat capacity change and does not conform to a simple rigid-body association. These contributions cannot come from conformational transitions of the cofactor, because they would be also seen in the interaction with the fast form. Rather, they come from conformational changes of the enzyme. Thrombomodulin induces a conformational change in thrombin that mimics the slow → fast transition, and the larger heat capacity change observed in the slow form contains the partial contribution of the Na^+-induced allosteric switch that is known to have a large and negative heat capacity change [79].

Further information on the energetics of the thrombin-thrombomodulin interaction has come from salt dependence studies. The value of K_d in 200 mM salt is remarkably different in NaCl, choline chloride and NaF (table 10). For example, in the case of hTM^+ and rTM the affinity changes by two orders of magnitude between choline chloride and NaF at the same ionic strength. This difference cannot depend on electrostatic screening, which is due only to the charge of the salt species present in solution. Rather, it signals the presence of specific binding interactions of Na^+ and Cl^- that are selectively suppressed in choline chloride and NaF because of the inert nature of choline and F^- [61, 62, 82, 83, 127]. Na^+ binding favors the formation of the thrombin-thrombomodulin complex, whereas Cl^- binding opposes it. The difference in the K_d values observed between NaCl and choline chloride is almost the same for all ligands and is due to the slow → fast transition of thrombin. On the other hand, the difference between NaCl and NaF depends strongly on the presence of the chondroitin sulfate moiety. Specific Cl^- binding to exosite I is supported by both experimental [61, 127] and computational studies [112] and explains the difference seen between NaCl and NaF for hTM^-, $TM^{4\text{-}5}$ and $TM^{4\text{-}5\text{-}6}$. The larger difference seen for hTM^+ and rTM suggests that additional Cl^- binding sites must be located in exosite II where the chondroitin sulfate moiety binds.

This scenario is supported by the more pronounced salt dependence of hTM^+ and rTM compared to hTM^- and the fragments. The value of Γ_{salt} (table 9) is -4.8 ± 0.6 for hTM^+ and rTM, but drops to -2.2 ± 0.4 for hTM^- and the two fragments $TM^{4\text{-}5}$ and $TM^{4\text{-}5\text{-}6}$. The chondroitin sulfate moiety contributes more than half the value of Γ_{salt}. The independence of Γ_{salt} on the particular salt used, as seen for the thrombin-fibrinogen interaction (table 8), shows that this parameter reflects predominantly the electrostatic screening of ions on the surface of the macromolecules. However, Γ_{salt} for hTM^+ far exceeds the values reported for fibrinogen [127] or hirudin [151] binding (tables 7 and 8), and signals a much larger electrostatic contribution to the binding of thrombomodulin to thrombin. The value of Γ_{salt} for thrombomodulin binding is unusually high for protein-protein interactions and suggests the involvement of a polyelectrolyte-like domain in the formation of the complex. The only domain capable of such behavior is the chondroitin sulfate moiety in view of its high content in spatially ordered negative charges. Studies on the interaction of heparin with thrombin have reported a value of Γ_{salt} of -4.8 [21], consistent with the polyelectrolyte-like nature of heparin. The results for hTM^+ and rTM, in conjunction with those obtained with heparin, strongly support an interaction of the chondroitin sulfate with exosite II, which is consistent with recent site-directed mutagenesis studies [65, 66].

An important aspect of thrombomodulin function pertains to the change in specificity of thrombin toward

Table 11. Effect of human thrombomodulin (TM) and the hirudin fragment 55–65 (hir^{55-65}) on the specificity constant $s = k_{cat}/K_m$ (in $\mu M^{-1}s^{-1}$) for the hydrolysis of synthetic substrates by thrombin[a].

	s	s +TM	s +hir^{55-65}	s	s +TM	s +hir^{55-65}	ΔG_c	ΔG_c +TM	ΔG_c +hir^{55-65}
	Fast form			Slow form					
FPR	90	94	117	3.0	20	21	−1.8	−0.7	−0.8
FPK	7.9	10	6.3	0.35	3.4	1.6	−1.8	−0.6	−0.8
FGR	2.0	2.1	1.7	0.86	4.4	3.1	−0.5	+0.4	+0.4
VPR	100	96	98	6.7	27	24	−1.6	−0.7	−0.8
FGK	0.021	0.035	0.023	0.0026	0.020	0.0084	−1.2	−0.3	−0.6
VPK	2.1	3.7	2.0	0.11	1.6	0.57	−1.7	−0.5	−0.7
VGR	0.34	0.44	0.32	0.17	0.73	0.66	−0.4	+0.3	+0.4
VGK	0.0047	0.010	0.0064	0.00079	0.0061	0.0027	−1.1	−0.3	−0.5
DPR	5.2	6.1	7.1	0.70	3.1	2.9	−1.2	−0.4	−0.5

[a]Errors are typically ±2%. Also listed are the values of coupling free energy for the slow→fast transition in the transition state, $\Delta G_c = -RT\ln r$ (in kcal/mol), where r is the ratio between the specificities of the fast and slow forms. Errors are typically ±0.1 kcal/mol.

protein C. It has been proposed that such an effect is borne out by a thrombomodulin-induced change in thrombin conformation [16]. Support for this comes from the fact that thrombomodulin binding induces changes in the environment of a chromophore covalently attached to the active site of thrombin [152]. This evidence, however, is purely qualitative as changes in the properties of a chromophore may occur independently of the changes necessary to alter thrombin specificity toward protein C. To sort out the effect of thrombomodulin on the active site of thrombin a detailed analysis has been carried out by Vindigni et al. [62] using the substrate library probing the S1, S2 and S3 sites of the enzyme (table 3).

When thrombomodulin binds to the fast form, there is at most a two-fold enhancement of specificity for all synthetic substrates including DPR, which mimics the sequence cut by thrombin in protein C (table 1). On the other hand, binding of thrombomodulin to the slow form produces a consistently higher increase in specificity by as much as 15-fold (table 11). The effects are similar for all substrates and cause a uniform reduction of the ΔG_c for allosteric switching. ΔG_c is the coupling free energy in the thermodynamic cycle that links the slow↔fast equilibrium to the binding of the substrate in the transition state and is a useful quantity to map the structural domains of the enzyme that are energetically linked to the allosteric equilibrium [37, 61, 98]. A reduced value of ΔG_c observed upon thrombomodulin binding demonstrates that this cofactor tends to abolish the differences between the slow and fast forms. The energetic contribution of this effect is about 1 kcal/mol for all substrates. This causes the slow form to become more specific in the case of substrates like FGR and VGR when thrombomodulin binds. The effect of thrombomodulin is energetically uniform on the S1, S2 and S3 specificity sites, and hence on all substrates probing these sites. The key observation, however, is that these effects are not peculiar to thrombomodulin, because they are also elicited in an almost identical manner by binding of hir^{55-65}.

The conclusion that thrombomodulin binding induces a conformational change of thrombin similar to the slow→fast transition is strongly supported by results obtained with the library of chromogenic substrates. Thrombomodulin has only a small effect on the fast form, whereas it enhances the specificity of the slow form up to 15-fold. As a result, binding of thrombomodulin drastically reduces the differences between the slow and fast forms. This is also seen with the natural substrate protein C, which is cleaved by the slow form with significantly higher specificity in the absence, but not in the presence of thrombomodulin [9, 23, 40, 153]. The effects on the synthetic substrates seen with thrombomodulin are also elicited by hir^{55-65}, whereas this ligand has no effect on the hydrolysis of protein C.

These results have a bearing on the mechanism that leads to the drastic (~500-fold) enhancement of thrombin specificity toward protein C upon thrombomodulin binding [16], which is seen in both the slow and fast forms [23]. The effect of thrombomodulin on the specificity sites S1, S2 and S3 of the enzyme produces a change in specificity that is either small (fast form) or at most 15-fold (slow form). Hence, thrombin must enhance its specificity toward protein C using sites other than those probed by the library of chromogenic substrates. Mutation of E192 to Gln enhances protein C cleavage in the absence of thrombomodulin [45]. It has been proposed that thrombomodulin alters the position of the side chain of E192, thereby relieving the unfavorable electrostatic coupling with D167 and D172 at the P3 and P3′ positions of protein C (table 1). Replacement of D167 with Phe and D172 with Asn in protein C enhances protein C activation by thrombin in the absence of thrombomodulin [46], just as seen for the E192Q mutant. Hence, the alternative possibility that

Table 12. Specificity of wild-type and mutant thrombins under physiological conditions[a].

	Slow form	Fast form	wt	R221aA	K224A	R221aA/K224A	Y225P	Δ E146-K149e
Na^+ binding[b] K_d (mM)	-	-	20 ± 1	140 ± 10	170 ± 20	700 ± 200	>900	280 ± 10
Fibrinopeptide A release k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	1.5 ± 0.1	35 ± 4	17 ± 1	1.1 ± 0.1	0.53 ± 0.04	0.017 ± 0.002	2.2 ± 0.1	0.071 ± 0.003
Fibrinopeptide B release k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.73 ± 0.04	17 ± 1	9.4 ± 0.5	0.49 ± 0.03	0.36 ± 0.04	0.014 ± 0.001	1.1 ± 0.1	0.018 ± 0.001
Protein C activation[c] k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	0.32 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.13 ± 0.01	0.080 ± 0.005	0.0080 ± 0.0004	1.6 ± 0.3	0.11 ± 0.01
Thrombomodulin K_d (nM)	5.3 ± 0.7	0.60 ± 0.02	0.99 ± 0.04	3.0 ± 0.1	8.2 ± 0.6	11 ± 1	3.8 ± 0.3	6.4 ± 0.1
Antithrombin III[d] k_{on} ($\mu M^{-1}s^{-1}$)	4.0 ± 0.2	20 ± 2	13 ± 1	1.1 ± 0.1	1.3 ± 0.1	0.27 ± 0.02	1.4 ± 0.1	0.55 ± 0.01
RAP[e]	16	0.5	1	9	12	36	56	120

[a]5 mM Tris, 145 mM NaCl, 0.1% PEG, pH 7.4, 37 °C. [b]5 mM Tris, I = 200 mM, 0.1% PEG, pH 8.0, 25 °C. [c]In the presence of 5 mM $CaCl_2$ and 100 nM human thrombomodulin. [d]In the presence of 0.5 USP/mL of heparin. [e]Relative anticoagulant potency calculated as the ratio of the rate for protein C activation over the rate for fibrinopeptide release relative to the same ratio of wild-type thrombin.

thrombomodulin actually moves D167 of protein C in the ternary complex, rather than E192 of thrombin, cannot be ruled out. E192 has been shown to make contacts with the charged amino group of substrates like those in table 3 [154] and therefore, if the side chain of this residue were displaced by binding of thrombomodulin, a significant change in specificity would be observed in the presence of this cofactor. Since no such change is detected when thrombin is in the fast form, and the effect in the slow form is due mainly to the slow → fast transition, there is little support for the hypothesis that thrombomodulin moves E192 of thrombin. Residues in the Na^+ binding loop affect protein C cleavage both directly and through the slow ↔ fast equilibrium [9, 67], while making no direct contacts with small chromogenic substrates. These residues may be responsible for the thrombomodulin-induced switch in specificity toward protein C. Furthermore, other mechanisms to enhance protein C cleavage by thrombin, independent of the fast → slow conversion and thrombomodulin binding, have recently been documented [153].

A more reasonable hypothesis is that thrombomodulin exerts its physiologically important function by influencing the conformation of the bound protein C in the thrombin-thrombomodulin-protein C ternary complex, thereby enhancing the specificity of the enzyme by turning protein C into a better substrate. A similar suggestion was originally made by Hayashi et al. [141]. A crucial component of this effect may be a movement of D167 of protein C, as implied by the observations of Richardson et al. [46]. It is extremely unlikely that the structural domains responsible for the enhancement in specificity are located entirely in regions of the enzyme other than critical sites within the catalytic pocket that can be probed with small chromogenic substrates. It is also extremely unlikely that thrombomodulin would induce a large conformational transition in thrombin and that this transition would not be detected experimentally as a large change in heat capacity as seen for the slow → fast transition [79] and many other cases [86]. Thrombomodulin makes extensive contacts with thrombin, bridging diametrically disposed regions on the surface of the enzyme like exosites I and II. The thrombin-thrombomodulin complex would therefore have the W60d loop and especially the Na^+ binding loop available for contacting protein C to form the ternary complex. It is conceivable that the bound protein C would make contacts with the bound thrombomodulin, perhaps at the level of the external portion of W60d loop of thrombin that is located in between exosites I and II. If this were the case, a chromogenic substrate contacting only the interior of the catalytic pocket would not experience the large change in specificity observed for protein C because it would lack the critical direct interaction with the cofactor. This would explain the similarity of effects seen on the chromogenic substrates with thrombomodulin and hir^{55-65}, but the lack of effect of hir^{55-65} on protein C hydrolysis.

This model predicts that it should be possible to find mutations of thrombomodulin which do not affect binding to thrombin but reduce the ability of thrombin, to cleave protein C. The fourth EGF-like domain of thrombomodulin is a prime candidate to test this prediction. It should also be possible to find mutations of protein C that affect cleavage by thrombin to different extents in the presence and absence of thrombomodulin. Grinnell et al. [155] have identified a cluster of positive charges in the primed sites of protein C at positions P5′ (K174), P8′ (R177) and P9′ (R178). Replacement of these residues with Glu produces a mutant protein C that is activated 16 times faster by thrombin in the absence, but not the presence, of thrombomodulin. They suggested that the cluster of positive charges may interact with thrombomodulin and in the absence of the cofactor may generate

electrostatic repulsion with the positively charged region of exosite I. More recently, Gerlitz and Grinnell [156] have documented mutations of the K37-K38-K39 sequence in protein C into the acidic DED and EEE sequences that practically obliterate the thrombomodulin-induced enhancement of protein C activation, without affecting the ability of free thrombin to cleave protein C. These important observations strengthen our hypothesis that the effect of thrombomodulin is predominantly on the bound protein C.

Finally, the hydrolysis of protein C by thrombin in the absence of Ca^{2+} and thrombomodulin occurs with rates that are comparable to those observed in the presence of Ca^{2+} and thrombomodulin [23, 39, 157]. Since Ca^{2+} has no effect on thrombin [23], the enzyme is perfectly competent to cleave protein C in the absence of thrombomodulin. Hence, the presence of acidic residues at P3 and P3′ does not necessarily result in a poor substrate for thrombin, as commonly believed. In fact, the thrombin receptor 1 (table 2) is a fairly good substrate despite the Asp residue at P3, and so are DPR (table 3) and similar chromogenic substrates with Asp at P3 [158]. Binding of Ca^{2+} changes the conformation of protein C and turns it into a very poor substrate. The effect of thrombomodulin is primarily on the bound protein C to override the negative influence of the divalent cation. The small (five-fold) enhancement of protein C cleavage seen in the absence of Ca^{2+} [23] is a good estimate of the true effect of thrombomodulin on thrombin. This enhancement is of the same magnitude as that observed with chromogenic substrates (table 11).

Anticoagulant thrombins

The dual role of thrombin as a procoagulant and anticoagulant factor opens the possibility of dissociating the two functions by *ad hoc* structural perturbations. Engineered thrombins, capable of cleaving only protein C, would act as more specific anticoagulants than warfarin or heparin [159]. Once administered in the blood, these mutants may help to maintain higher levels of activated protein C and thereby depress the coagulation response. The same effect would be difficult to achieve with direct infusion of activated protein C because of the limited life of this enzyme [160]. Further optimization of the anticoagulant properties can be obtained by a reduced inhibition by antithrombin III coupled with an insignificant activation of the thrombin receptors. In principle, an anticoagulant thrombin should have no activity toward fibrinogen or the thrombin receptors. In practice, due to scavenging of thrombin by antithrombin III in the blood that normally takes about 10–15 min [22], a mutant with clotting activity reduced by nearly 100-fold would work *de facto* as an exclusive activator of protein C. Mutations that tip the balance between procoagulant and anticoagulant activities of thrombin also bring about important clinical considerations because they may be associated with phenotypes with enhanced thrombotic or bleeding disorders.

Sadler et al. were the first to prove that fibrinogen and thrombomodulin binding could be differentially affected by site-directed mutations of exosite I and the W60d loop [39]. They found that charge reversal of R75 with Glu affects thrombomodulin binding more than fibrinogen and mutation of K60f to Glu does the reverse. This discovery has fostered the systematic search of epitopes that would differentially affect fibrinogen and thrombomodulin binding [135]. The search has led to the identification of a residue, E217, the mutation of which to Ala reduces fibrinogen binding 40-fold, whereas it has only a two-fold decrease on the rate of cleavage of protein C in the presence of thrombomodulin [74]. In the crystal structure [94], the Cγ of E217 makes a van der Waals contact with the carbonyl O atom of G12 of the fibrinogen Aα chain. Both E217 of thrombin and G12 of fibrinogen are highly conserved in different species. This has led Gibbs et al. to conclude that E217 plays an important role in fibrinogen recognition [74]. However, it is puzzling that a tenuous van der Waals contact would be responsible for nearly 2 kcal/mol of binding free energy of fibrinogen to thrombin. The crystal structure is probably missing important information on the interaction of thrombin with fibrinogen, because it lacks the contribution of the N-terminal domains of the Bβ and γ chains that may contribute to recognition of the physiological substrate. But careful consideration of the role of E217 in thrombin and G12 in fibrinogen may resolve the puzzle. G12 of fibrinogen is an important component of the β-turn of the Aα chain that enables F8 to re-enter the catalytic site of thrombin and make a favorable edge-to-face interaction with W215. Any other residue at this position would reduce the flexibility of the chain and compromise this important interaction. E217, on the other hand, is ion paired to K224 which coordinates the bound Na^+ through its carbonyl O atom [67, 68]. Disruption of the ion pair with the K224A substitution perturbs Na^+ binding and shifts the allosteric equilibrium toward the slow form, thereby reducing clotting without affecting significantly protein C activation. Not surprisingly, the properties of mutant K224A are practically identical with E217A [9]. Conservation of E217 is therefore due to the importance of this residue in forming the K224-E217 ion pair.

Following E217A, a number of mutations have been reported that tip the balance of thrombin specificity toward its anticoagulant activity. All these mutations, including E217A, have the underlying property of reducing Na^+ binding and stabilizing the anticoagulant slow form (table 12). The anticoagulant potency is measured relative to wild-type, under physiological conditions, as the ratio between the rates of activation of protein C and release of fibrinopeptide A. The slow form has a relative

anticoagulant potency of 16. Mutation of R221a and K224 reduces Na^+ binding and enhances the anticoagulant potency of the enzyme. Replacement of Y225 with Pro, the residue found in the serine proteases devoid of allosteric properties [8], results in complete obliteration of Na^+ binding and stabilization of the slow form with a consequent reduction of fibrinogen clotting and enhancement of protein C cleavage [9]. The effects observed with these mutations are purely allosteric, and are mediated by the slow↔fast equilibrium because they involve residues that do not make contacts with the fibrinopeptide A according to the crystal structure [94, 95]. The drastic reduction in fibrinogen clotting observed in the double mutant R221aA/K224A cannot be interpreted solely as a perturbation of the slow↔fast equilibrium, but implies additional structural changes and perhaps direct perturbation of fibrinogen binding. Mutations in the Na^+ binding loop therefore compromise fibrinogen clotting more than protein C activation by weakening Na^+ binding and possibly interfering directly with fibrinogen binding.

Although perturbation of Na^+ binding is the dominant factor in tipping the balance of thrombin specificity towards protein C, an alternative way of selectively interfering with fibrinogen binding has been revealed by a thrombin modulator that inhibits fibrinogen clotting and enhances protein C activation independent of Na^+ or thrombomodulin binding [153]. The structural domain of thrombin responsible for the effect has not been identified, but this seminal observation suggests that regions of the enzyme not involved directly in Na^+ or thrombomodulin binding may serve as epitopes for fibrinogen, but not protein C, recognition. A crucial implication of this observation is that these regions, once identified, may provide targets for a new class of anticoagulants. An active site inhibitor of thrombin would shut down both procoagulant and anticoagulant activities, whereas a drug that selectively suppresses fibrinogen binding would have an enhanced anticoagulant potency in view of the lack of inhibition of the protein C-dependent anticoagulant pathway [161].

The autolysis loop is strategically located in between the Na^+ binding site and exosite I (fig. 1), which provides a site for fibrinogen and thrombomodulin binding. The loop spans nine residues, from E146 to K149e, and distinguishes itself from the homologous region in other serine proteases like trypsin and chymotrypsin for the five residue insertion A149a–K149e. Little importance has been attributed to this loop because enzymatic cleavage at various positions is inconsequential for thrombin function [41, 42] and its conformation changes depending on solution conditions and crystal packing [24, 54]. Inspection of the sequence of the autolysis loop in thrombin from different species [70] reveals a lack of conservation for the five residue insertion A149a–K149e. Deletion of the A149a–K149e five residue insertion produces no significant change in the functional properties of thrombin [44]. On the other hand, the sequence E146-T147-W148 preceding the insertion is highly conserved. Both E146 and W148 are present in all species, from human to hagfish [70]. Conservation of W148 is unclear because mutation of this residue has no effect on thrombin function [37]. Conservation of E146, on the other hand, is justified by the important ion pair formed with R221a in the Na^+ binding loop [9]. Deletion of the E146-W148 segment alone reduces fibrinogen clotting 20-fold and protein C activation 16-fold [162]. Deletion of all nine residues E146-K149e results in a mutant that has practically lost its clotting activity because of the remarkable reduction in the release of fibrinopeptides A and B by 240-fold and 520-fold respectively, whereas the ability to activate protein C is reduced only two-fold (table 12). Binding of thrombomodulin is reduced six-fold, inhibition by antithrombin III is reduced 20-fold and Na^+ binding is reduced over ten-fold [44].

Some of the properties of the loopless mutant, like the interaction with thrombomodulin, antithrombin III and protein C, can be explained in terms of a perturbation of the slow↔fast equilibrium due to loss of the R221a-E146 ion pair as for the R221aA mutant. In fact, the loopless mutant binds Na^+ very weakly. The effect on fibrinogen, on the other hand, cannot be reconciled entirely with a perturbation of the slow↔fast equilibrium in favor of the slow form. Most of the effect must originate from direct perturbation of fibrinogen binding due to the loss of critical interactions in the loopless mutant. Because none of the residues deleted in this mutant makes contact with fibrinopeptide A, other portions of the fibrinogen molecule must interact with the autolysis loop. The possibility that the conformation of other regions of thrombin interacting exclusively with fibrinogen is altered in the loopless mutant cannot be ruled out, although the modest effect seen for protein C, antithrombin III and thrombomodulin binding excludes a drastic perturbation of thrombin structure.

Independent of whether the autolysis loop participates directly in fibrinogen binding or influences other regions of the enzyme, deletion of the loop selectively suppresses fibrinogen clotting. The 240-fold reduction in the release of fibrinopeptide A with a concomitant 2-fold loss of protein C activation implies that use of this mutant in vivo at twice the concentration as wild-type thrombin would generate the same amount of activated protein C, but would clot blood in 8 hours instead of 4 minutes [22]. Given the limited life of thrombin in the blood due to scavenging by antithrombin III [22], the loopless mutant is *de facto* an exclusive activator of protein C. Its properties are extraordinarily similar to those of the E217K mutant recently engineered for optimal anticoagulant activity in vivo [160] and represent a substantial gain in anticoagulant potency compared to other single-

Table 13. Classification of serine proteases based on the codon for S195 and residue 225[a].

	Protease	Sequence 220–226	Residue 225	S195 codon		Protease	Sequence 220–226	Residue 225	S195 codon
A. TCN/P225 lineage									
1.	7S NGF-α (m)	CGEPTEPS	P	TCN	32.	Tissue kallikrein	CGTPNKPS	P	TCN
2.	7S NGF-γ (m)	CGEPKKPG	P	TCN	33.	Tonin (r)	CAKPKTPA	P	TCN
3.	γ-Renin (m)	CGIPGVSA	S	TCN	34.	tPA	CGQKDVPG	P	TCN
4.	Ancrod (s)	CAQPDKPA	P	TCN	35.	Trypsin	CAQKNKPG	P	TCN
5.	Arginine esterase (d)	CGKPQMPS	P	TCN	36.	Trypsin-like protease (b)	CARSGVPG	P	TCN
6.	Batroxobin (s)	CAEPRKPA	P	TCN	37.	Tryptase	CAQPNRPG	P	TCN
7.	Chymotrypsin	CS-TSSPG	P	TCN	38.	Urokinase	CALKDKPG	P	TCN
8.	Clotting enzyme (c)	CALPGFPG	P	TCN	B.	TCN/X225			
9.	Easter (ff)	CGLAGWPG	P	TCN					
10.	Elastase I	CNVTRKPT	P	TCN	1.	C2	CHGSSNKN	K	TCN
11.	Elastase II	CNYYHKPS	P	TCN	2.	Ra-reactive factor	CGKKDRYG	Y	TCN
12.	Elastase IIIa	CNFIWKPT	P	TCN	3.	Serine protease (ff)	CARPQEFG	F	TCN
13.	Elastase IIIb	CNTRRKPT	P	TCN	C.	AGY/Y225 lineage			
14.	Enterokinase	CALPNRPG	P	TCN					
15.	Factor B	CKNQKRQK	Q	TCN	1.	Activated protein C	CGLLHNYG	Y	AGY
16.	Factor D	CGNRKKPG	P	TCN	2.	C1r	CSRGYGFY	F	AGY
17.	Factor I	CGKPEFPG	P	TCN	3.	C1s	CGTYGLYT	Y	AGY
18.	Factor XIa	CAQRERPG	P	TCN	4.	CASP (h)	CGTYGIYT	Y	AGY
19.	Factor XIIa	CGDRNKPG	P	TCN	5.	Clotting factor C (c)	CGKANQYG	Y	AGY
20.	Granzyme A	CGDPRGPG	P	TCN	6.	Factor VIIa	CATVGHFG	F	AGY
21.	Hypodermin A (i)	CARPSYPG	P	TCN	7.	Factor IXa	CAMKGKYG	Y	AGY
22.	Hypodermin B (i)	CAYPGFPG	P	TCN	8.	Factor Xa	CARKGKYG	Y	AGY
23.	Leukocyte elastase	CASGLYPD	P	TCN	9.	MASP-2	CGEAGQYG	Y	AGY
24.	Myeloblastin	CATRLFPD	P	TCN	10.	Thrombin	CDRDGKYG	Y	AGY
25.	Plasma kallikrein	CARREQPG	P	TCN	D.	AGY/X225			
26.	Proteinase B (b)	CSSGGTTF	T	TCN					
27.	Semenogelase	CALPERPS	P	TCN	1.	Acrosin	CALAKRPG	P	AGY
28.	Serine protease (i)	CG-SASPD	P	TCN	2.	Hepsin	CALAQKPG	P	AGY
29.	Serine protease (b)	CS-TTTPA	P	TCN	3.	Plasmin	CARPNKPG	P	AGY
30.	Snake gene (ff)	CAAPNAPG	P	TCN	4.	Proteinase A (b)	CRTGGTTF	T	AGY
31.	Stubble gene (ff)	CAEANLPG	P	TCN					

[a]Listed are all the different serine proteases of the chymotrypsin family [1, 2]. For proteases present in multiple species (e.g. trypsin and thrombin), only the human form is listed to avoid redundancy. Thrombin consistently has Y225 in different species. Trypsin has P225 in all species with the only exception of *Fusarium oxysporum*, which has S225. All proteases are human unless otherwise indicated by parentheses: b = bacterium, c = crab, d = dog, ff = fruit fly, h = hamster, i = insect, m = mouse, r = rat, s = snake. The TCN/P225 lineage comprises P225 and all residues within one mutational step from Pro in the genetic code, whereas the TCN/X225 lineage includes any residue at position 225 requiring more than one mutational step from Pro in the genetic code. The AGY/Y225 lineage comprises Y225 and all residues within one mutational step from Tyr in the genetic code, whereas the AGY/X225 lineage includes any residue at position 225 requiring more than one mutational step from Tyr in the genetic code.

site substitutions [9, 74]. The autolysis loop therefore represents an ideal target for molecules aimed exclusively at suppressing the procoagulant activity of the enzyme. Targeting this loop, more conspicuous in thrombin than trypsin, should also reduce the cross-reactivity of the modulator with the digestive protease that so often compromises the biological potency of active-site inhibitors of thrombin designed for oral availability [163].

Na^+-induced allosteric regulation in serine proteases

The Na^+ effect on thrombin is an example of a general regulation mechanism for enzyme activity by monovalent cations first described by Boyer for pyruvate kinase [164]. He found that the catalytic activity of pyruvate kinase was maximal in the presence of K^+ and NH_4^+, but would decrease substantially in the presence of Li^+ and Na^+. Following this seminal observation, almost a hundred enzymes were found to be activated specifically by K^+ [165, 166]. These enzymes catalyse mainly phosphoryl transfer reactions and work inside the cell where the concentration of K^+ is high. Suelter speculated that enzymes working outside the cell would, on the other hand, exploit Na^+ as a possible activator, but with the exception of oxalacetate decarboxylase, no other examples of Na^+-activated enzymes could be described at the time [80].

The earliest report of specific monovalent cation effects on enzymes involved in blood coagulation dealt with the differential effect of Na^+ and K^+ on the esterolytic activity of thrombin and specifically the higher efficacy of K^+ in promoting this activity [167, 168]. Orthner and Kosow [77, 169] subsequently proved that factor Xa and thrombin cleave chromogenic amide substrates with higher specificity in the presence of Na^+. Castel-

lino reported similar effects for activated protein C [170, 171]. That the Na^+-activation of thrombin is a consequence of Na^+-induced allosteric transitions of the enzyme was unequivocally established by Wells and Di Cera [7]. This discovery, along with that of the change in thrombin specificity induced by Na^+ binding [23], firmly established the importance of Na^+ in protease regulation and set the stage for the analysis of similar effects in other blood clotting enzymes and serine proteases in general.

The availability of several crystal structures documenting the molecular basis for monovalent cation activation of enzymes has renewed interest in this mechanism of regulation. Monovalent cation binding sites have recently been described for dialkylglycine decarboxylase [172], pyruvate kinase [173], the molecular chaperone Hsc70 [174], fructose-1,6-bisphosphatase [175], S-adenosylmethionine synthetase [176] and Trp synthase [177]. Monovalent cations affect the properties of an enzyme in essentially two ways. One is by forming a ternary complex with the enzyme and the substrate. In this case, the requirement for the monovalent cation is absolute and no substrate hydrolysis is observed in its absence. This mode of action is predominant among K^+-activated enzymes as documented by the molecular chaperone Hsc70 [178]. The alternative mode of action is through allosteric activation induced by binding to a site distinct from the active site. This mode of action is documented in only a few K^+-activated enzymes, like pyruvate kinase [173], but seems to be preponderant among the Na^+-activated enzymes [67, 68].

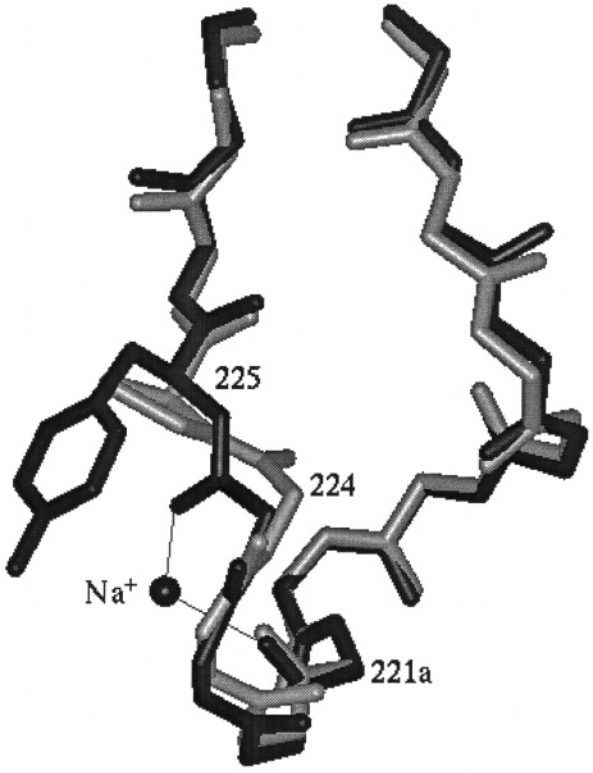

Figure 5. Comparison of the Na^+ binding loop of thrombin (black) with the homologous region of trypsin (gray). In thrombin, the carbonyl O atoms of R221a and K224 contribute two of the six ligating groups in the octahedral coordination shell of the bound Na^+, which is completed by four water molecules (not shown, see fig. 2). In trypsin, the presence of P225, as opposed to Y225 in thrombin, reorients the carbonyl O atom of residue 224 in a position that is incompatible with Na^+ binding.

In the case of thrombin, identification of the allosteric Na^+ binding site [67] has been instrumental in the elucidation of the molecular basis of the Na^+ regulation in the entire class of serine proteases of the chymotrypsin family. The Na^+-induced switch in specificity from protein C to fibrinogen discovered for thrombin [23] represents a novel mode of protease regulation and raises the question of its generality among related enzymes. We have demonstrated that some proteases involved in blood coagulation and the complement cascade require Na^+ for optimal catalytic activity, whereas proteases involved in digestive processes and fibrinolysis do not [8]. The molecular origin of this difference is remarkably simple and provides one of most important structure-function correlations ever reported for serine proteases or enzymes in general.

The region spanning residues 215–227 and comprising the Na^+ binding loop in thrombin bears very little homology among different serine proteases (table 13), but the backbone architecture of the loop appears to be highly conserved (fig. 5), except for one notable feature. In thrombin, the carbonyl O atoms of K224 and R221a provide two of the six ligating groups in the octahedral coordination shell of the bound Na^+. In trypsin, the presence of P225 constrains the carbonyl O atom of residue 224 to reorient in a manner incompatible with Na^+ coordination. Indeed, there is no evidence of bound Na^+ in the structure of trypsin [47]. In the crystal structures of pancreatic elastase and plasma kallikrein, that like trypsin have P225, the carbonyl O atom of residue 224 is similarly oriented and there is no evidence of bound Na^+. On the other hand, the carbonyl O atom of residue 224 in factor Xa, that like thrombin has Y225, is correctly positioned for Na^+ coordination and the refined crystal structure documents a bound Na^+ in nearly the same position as in thrombin [68, 179]. Analysis of the complete set of sequences of serine proteases of the chymotrypsin family [1, 2] shows that residue 225 is, remarkably, either Pro or Tyr in 47 out of a total of 55 different proteases (table 13). When the catalytic activity of a number of serine proteases was studied, those with Y225 or F225 were found to discriminate among monovalent cations and show maximal catalytic activity in the presence of Na^+. On the other hand, proteases with P225 showed no discrimination among monovalent cations, which indicates the lack of specific

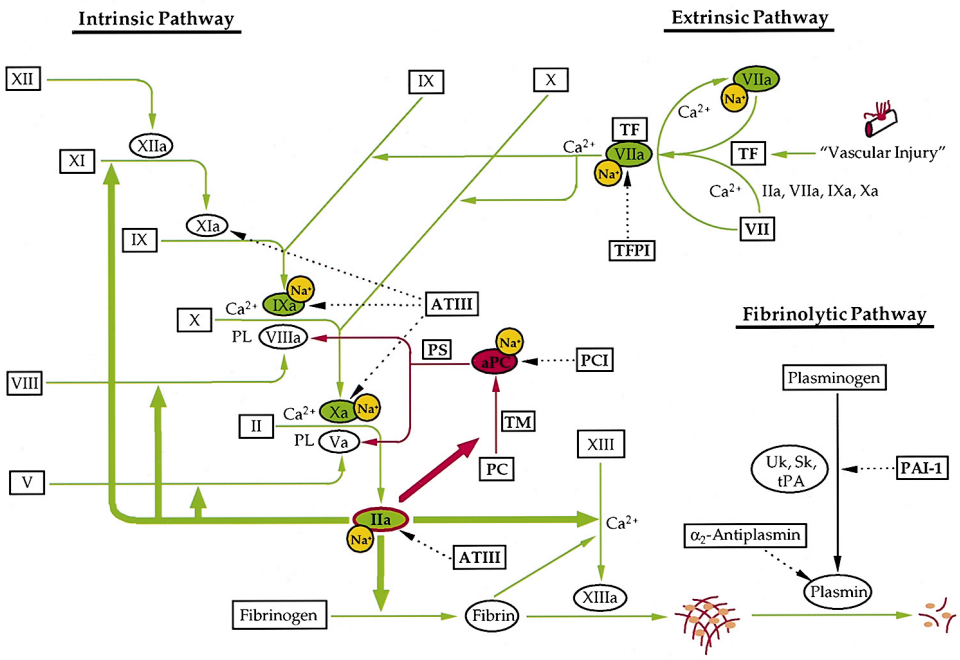

Figure 6. Schematic representation of the blood coagulation cascade showing the major interactions and regulatory feedbacks that lead to the conversion of zymogens (rectangles) to active proteases (ovals). Also shown is the fibrinolytic pathway leading to digestion of the fibrin clot. Procoagulant reactions are in green, reactions with protease inhibitors are black dotted lines, and the protein C mediated anticoagulant pathway is in red. The vitamin K-dependent proteases thrombin (factor IIa) , factors Xa, IXa, VIIa and activated protein C all possess a functional Na^+ binding site (yellow) that is required for efficient catalysis. These proteases occupy strategic positions in the cascade and define the intersection between the intrinsic and extrinsic pathways. Na^+ may well be regarded as a coagulation factor that guarantees rapid activation of factor X by factor XIa and VIIa, with a consequent rapid generation of thrombin from prothrombin (factor II) and efficient cleavage of fibrinogen by the fast form of thrombin. Abbreviations: aPC, activated protein C; ATIII, antithrombin III; PAI-1, plasminogen activator inhibitor type 1; PC, protein C; PCI, protein C inhibitor; PL, phospholipids; PS, protein S; Sk, streptokinase; TF, tissue factor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; tPA, tissue plasminogen activator; Uk, urokinase.

binding interactions of Na^+, Li^+ or K^+. Replacement of Y225 with Pro in thrombin results in the loss of discrimination among monovalent cations and allosteric regulation and stabilization of the anticoagulant slow form (table 12). Hence, residue 225 determines the Na^+-induced allosteric enhancement of catalytic activity in serine proteases of the chymotrypsin family [8].

When the Na^+-dependent allosteric regulation of thrombin was discovered [7], it was expected that a similar effect would be present in other blood clotting enzymes based on the early proposal by Suelter that enzymes activated by K^+ are intracellular, whereas those requiring Na^+ would work in extracellular environments [80]. The important role of residue 225, however, indicates that the basis for Na^+ binding in serine proteases is determined by the residue present at position 225 and not by the specific function of the enzyme. In fact, coagulation factor XIa, plasmin and tissue plasminogen activator, that carry P225, do not bind Na^+ although they are active in the blood like thrombin, factor Xa and C1r. The presence of Pro is sufficient to obliterate Na^+ binding, because of the constraints introduced in the protein backbone around residue 224. It can be predicted that proteases with P225 will show no effect of Na^+ on their catalytic activity. On the other hand, if residue 225 is not a Pro, one cannot easily conclude that the enzyme will bind Na^+ because other factors certainly influence the Na^+ affinity [9, 44, 67]. If elimination of Pro at position 225 were not only necessary but also sufficient for Na^+ binding, the residue at position 225 would be inconsequential.

In contrast, there is a strong preference for Tyr or Phe at this position in Na^+-activated proteases. The Pro → Tyr substitution requires two mutational steps in the genetic code. Hence, introduction of a functional Na^+ site with Y225 was a key event in the molecular evolution of serine proteases. In thrombin, Y225 is absolutely conserved from hagfish to human [70], two species 450 million years apart in evolutionary terms [4]. Tyr or Phe at position 225 must play a stabilizing role, either by providing a favorable cation-π interaction between the aromatic ring and the bound cation [180], or by screening solvent molecules from the buried cavity hosting the metal ion. If so, considerable specificity should be lost

with residues like Ala or Ser at position 225. The role of Y225 in thrombin is being tested by systematic site-directed mutagenesis.

The most exciting possibility brought about by the discovery of the role of residue 225 is the introduction of the Na^+-induced allosteric enhancement of catalytic activity in proteases carrying P225. These enzymes include biologically and pharmacologically important proteases like plasmin and tissue plasminogen activator. Na^+ binding may endow these proteases with allosteric regulation and enhanced catalytic properties that would be highly desirable in practical applications. Evidence that introducing Na^+ binding may require *ad hoc* changes in different proteases comes from the observation that the simple mutation P225S in Easter is associated with a phenotype consistent with enhanced catalytic activity of the protease [181], but the same replacement in tissue plasminogen activator, as well as the P225Y replacement, do not result in Na^+ binding (Edwin Madison, personal communication). This reinforces the importance of identifying the structural factors that control Na^+ binding in thrombin in order to gain a predictive understanding of the changes that need to be introduced in proteases devoid of Na^+ binding to enable the Na^+-dependent allosteric enhancement of catalytic activity.

Residue 225 also shows an intriguing correlation with the codon used for S195 in the active site (table 13). Either the TCN or AGY codon are used for this residue [182]. With only few exceptions, serine proteases using a TCN codon for S195 have P225, whereas those with Y225 use an AGY codon. This suggests that serine proteases may have evolved from two main lineages: a TCN/P225 lineage with a trypsin-like ancestor and an AGY/Y225 lineage with a thrombin-like ancestor [8]. It has been suggested that the AGY codon evolved at different stages from the TCN codon [183, 184], because this codon is consistently found in digestive proteases present in archaea, whereas proteases using the AGY codon are typically involved in exclusive functions of vertebrates such as blood coagulation, fibrinolysis and complement. The AGY/Y225 lineage likely evolved from the TCN/P225 lineage. Both the TCN → AGY and Pro → Tyr conversions require at least two mutational events in the genetic code and might have occurred in parallel. Proteinases A and B from *Streptomyces griseus* both contain a Thr at position 225, but one has a TCN codon for S195 whereas the other has an AGY codon. These enzymes resemble mammalian enzymes more than expected, causing disagreement about the actual evolutionary relationship of the trypsins. Hartley [185] proposed a process involving gene transfer from a higher organism to explain this curiosity. However, Hewett-Emmet et al. [186] were able to construct an evolutionary tree with the bacterial enzyme at the root of the tree. Hence, the evolutionary pressure to mutate P225 in the ancestral TCN/P225 protease might have preceded that to mutate the codon for S195. Consistent with this hypothesis, the trypsin from *Fusarium oxysporum* has a TCN codon for S195 but a Ser at position 225. In the crystal structure [187] the carbonyl O atom of residue 224 assumes a position intermediate between the carbonyl O atom of bovine trypsin, with P225, and thrombin, with Y225. This suggests that the nature of the side chain at position 225 may influence the position of the carbonyl O atom of residue 224 for correct Na^+ coordination.

An important intermediate along the TCN → AGY pathway is the TGY codon for Cys. Cysteine proteases might have originated as a result of the codon conversion of the serine. However, of the two possible intermediates along the Pro → Tyr pathway for residue 225, Ser and His, only Ser is documented in the currently available sequences of serine proteases. The intermediate lineages TCN/X225 and AGY/X225 (table 13) have Lys or Thr at position 225 and offer few clues as to what might have been the direct pathway from the ancestral protease to the vitamin K-dependent proteases and the related complement proteases C1r, C1s and the newly discovered MASP-2 [188]. The divergence of vitamin K-dependent coagulation factors is estimated to have occurred some 600 million years ago, which coincides with the divergence of the fibrinogen γ and β chains [4]. Sorting out these evolutionary pathways is of great interest as it will provide new insight into the development of complex systems from simple ones. In addition, this information may facilitate current understanding of physiological processes and their interactions.

The novel classification of serine proteases of the chymotrypsin family given in table 13 is based on the Na^+-induced allosteric regulation, which represents a new and important aspect of protease function. An obvious question is why the need for a Na^+-dependent allosteric enhancement of catalytic activity emerged during evolution of this class of enzymes. One possibility is that a functional Na^+ site might have been incorporated into the structure of an ancestral protease to gain catalytic activity only when the enzyme was secreted from the cell. Such a mechanism would have preceded the more efficient zymogen → enzyme conversion seen for almost all proteases and perhaps is still present in some primitive organisms. Another possibility is that Na^+-activated proteases would be involved in cell damage, to respond to a transient increase in the intracellular Na^+ concentration. The Na^+ binding site would then be retained during evolution to guarantee high catalytic efficiency under conditions where the concentration of Na^+ is high, as in the extracellular fluids. Interestingly, the Na^+-sensitive proteases are all involved in blood coagulation and complement, two functions that were once indistinguishable and that evolved from a primordial mechanism of defense [5, 6], still present in some species like the horseshoe crab [189]. The Na^+-activated

proteases C1r, C1s, MASP-1 (Ra reactive factor) and MASP-2 occupy a prominent position in the complement system. This system can be activated in three ways: by the classical pathway initiated by antigen-antibody complexes and propagated by C1r and C1s, by the alternative pathway initiated by direct contact with structures on microbial surfaces, and by an antibody-independent pathway sustained by newly discovered mannan-binding lectins MASP-1 and MASP-2 [188]. The high homology between thrombin, the vitamin K-dependent factors, and these complement proteases suggest divergent evolution from a common thrombin-like ancestor that was once involved in triggering clot formation as well as mediating the response to microbial invaders. The discovery of Na^+ binding to serine proteases and its molecular origin imply that some of the major factors that bring about blood coagulation possess a functional Na^+ site and are specifically activated by this monovalent cation [8]. A schematic representation of the coagulation cascade incorporating the role of Na^+ is given in figure 6. The vitamin K-dependent, Na^+-activated proteases occupy critical positions in the cascade. This implies that Na^+ may play a key role not only in the conversion of fibrinogen into fibrin by thrombin, but also in the generation of thrombin from prothrombin by factor Xa and the activation of factor X by factors IXa and VIIa in the intrinsic and extrinsic pathways respectively. Although the effects of Na^+ on the physiologically relevant interactions of factors VIIa, IXa and Xa have not yet been characterized, they are expected to be large and significant. The recent identification of the epitopes for tissue factor binding on factor VIIa [190], involving residues of the Na^+ binding loop, points to the likelihood of a large Na^+ effect on the activation of factor X. Similarly, the anticoagulant action of activated protein C may be expected to depend strongly on the availability of Na^+. As more information is gathered on all these interactions, the prominent role of Na^+ in the coagulation cascade and the complement system will become obvious. Like Ca^{2+}, Na^+ may well be regarded as a coagulation factor necessary for efficient activation of zymogens in the cascade. In addition, the allosteric equilibrium between the Na^+-free and Na^+-bound forms of each protease would offer a basic molecular mechanism for fine regulation.

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