Determinants of Specificity of Factor Xa Interaction with its Physiological Inhibitors

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Abstract: Factor Xa (fXa) is the vitamin K-dependent serine protease of the prothrombinase complex (fXa, factor Va, negatively charged membrane, and calcium) which is responsible for the conversion of prothrombin to thrombin in the final stage of the coagulation cascade. The proteolytic activity of fXa in plasma is primarily regulated by three physiological inhibitors, antithrombin (AT), protein Z-dependent protease inhibitor (ZPI) and tissue factor pathway inhibitor (TFPI). The first two inhibitors belong to the serpin family of plasma inhibitors, both of which require cofactors for their effective interaction with fXa. Thus, the AT interaction with the heparin-like glycosaminoglycans on the surface of the endothelium, and the ZPI complex formation with protein Z on membrane phospholipids is required for the physiological regulation of fXa by both serpins. On the other hand, TFPI is a slow and tight-binding, Kunitz type inhibitor that is capable of rapidly inhibiting fXa independent of a cofactor. This article will review the structural features that enable fXa to specifically interact with these three inhibitors under different conditions.

Keywords: Factor Xa, antithrombin, PZ-dependent protease inhibitor, Tissue factor pathway inhibitor, Serpin, Kunitz inhibitor.

INTRODUCTION

Factor Xa (fXa) is a vitamin K-dependent coagulation serine protease, which, upon binding to its cofactor factor Va (fVa) on membrane surfaces in the presence of Ca^{2+} ions, rapidly activates prothrombin to thrombin in the clotting cascade [1-4]. The activation complex, called prothrombinase, activates prothrombin with a catalytic efficiency that is more than 10^5-fold greater than that of fXa alone [1,2]. FXa circulates in plasma as a light and heavy chain molecule held together by a disulfide bond [5]. The light chain of fXa contains the non-catalytic γ-carboxyglutamic (Gla) and two epidermal growth factor-like domains (EGF1 and EGF2) that are conserved in all other vitamin K-dependent plasma serine proteases including factors VIIa (fVIIa), IXa (fIXa) and activated protein C (APC) [3]. The N-terminal Gla domain of fXa contains eleven Gla residues, which are required for the Ca^{2+}-dependent binding of the protease to fVa on negatively charged membrane surfaces with high affinity [1-3,6,7]. The role of EGF domains in the function of fXa is not well known. However, the EGF1 domain is known to have a functionally significant Ca^{2+}-binding site [5]. The catalytic domain of fXa with a trypsin-like primary specificity pocket is located on the C-terminal heavy chain of the molecule [8]. The catalytic groove of fXa (and other coagulation proteases) is surrounded by several surface loops including 39-loop, autolysis loop (residues 143-154), 60-loop, and the Na^+-binding 225-loop (chymotrypsinogen numbering system has been used throughout the manuscript [9]), all of which play key roles in determining the exosite-binding specificity of the protease in reaction with its physiological substrates and inhibitors [10-12]. Recent crystal structure determination of the human Gla-domainless fXa (GD-fXa) suggests that the EGF2 domain is in intimate contact with the catalytic domain and that the two domains constitute a single functional unit [8]. The binding of the Gla and EGF domains of fX/fXa to their target cofactors on membrane surfaces may modulate the structure and physiological function of fX/fXa in the clotting cascade [13]. Like other vitamin K-dependent coagulation proteases, fXa contains a single Ca^{2+}-binding site on the catalytic domain on a conserved surface loop spanning residues 70-80 and a Na^+-binding site on the conserved 225-loop [14,15]. The occupancy of these sites with the respective metal ions is required for the structural integrity and the catalytic function of fXa in plasma [15].

Since fXa is the point at which both intrinsic and extrinsic pathways of coagulation converge, its regulation by plasma inhibitors is critical for maintenance of hemostasis. At least three different physiological inhibitors, antithrombin (AT), protein Z-dependent protease inhibitor (ZPI) and tissue factor pathway inhibitor (TFPI) regulate the proteolytic activity of fXa in plasma. The first two inhibitors belong to the serine protease inhibitor (serpin) family of plasma inhibitors, both of which require cofactors for their effective interaction with fXa [16,17]. On the other hand, TFPI is a Kunitz-type inhibitor that binds tightly to fXa and can rapidly inhibit the protease activity independent of a cofactor [18]. Other plasma inhibitors that are known to regulate the activity of fXa include α_2-macroglobulin and α_1-proteinase inhibitor [19]. In particular, α_2-macroglobulin has been reported to play a key role in regulating fXa in plasma as it targets fXa for the hepatic clearance by the low-density lipoprotein receptor-related protein (LRP) [19]. However, this article will only review the structural features that enable fXa to specifically interact with AT, ZPI and TFPI.

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A. INHIBITION BY AT

AT (I04.018), with plasma concentration of ~2.3 μM is the primary serpin inhibitor of fXa and other proteases of the intrinsic and extrinsic blood coagulation cascade [16]. Similar to other serpins, the structure of AT contains three β-sheets (A to C) and nine α-helices (A to I) [20,21]. The reactive center loop (RCL) that docks into the active site groove of target proteases, extends from P15 to P5' (nomenclature of Schechter and Berger [22]) and connects the large 5-stranded A-sheet to the smaller 3-stranded C-sheet [20,21]. AT inhibits fXa and other coagulation proteases by a branched pathway, suicide substrate inhibition mechanism in which a Michaelis-type enzyme-inhibitor complex, formed in the first reaction step, is converted to a covalent acyl-enzyme intermediate complex in the second step of the reaction [23]. Similar to interaction with true substrates, a typical salt-bridge interaction between Asp-189 at the primary specificity pocket of fXa and an Arg at the P1 position of RCL accounts for the specificity of the initial interaction of the protease with the inhibitor [8,23,24]. However, unlike reaction with true substrates, attack of the P1-Arg by the catalytic Ser-195 in the second step induces a conformational change in RCL that leads to insertion of the loop into β-sheet A, thereby relocating the protease to the opposite end of the molecule [25,26]. Loop insertion disrupts the catalytic machinery of the enzyme so that the intermediate is deacylated very slowly ($t_{1/2}$ 0.5-3 days), and thus trapped as a kinetically stable inactive complex [23,26,27]. It should be noted that the covalent fXa-AT complex has a much shorter half-life in plasma (2-5 min) since it is eliminated from circulation via the LRP-mediated uptake by hepatic cells [19]. In addition to P1-Arg, structural and mutagenesis data have indicated that a Gly at the P2 position of RCL is also required for an effective interaction of AT with fXa [8,28]. The requirement for a small residue at the P2 position of the serpin appears to be due to the presence of two bulky residues Tyr-99 and Phe-174 at the P2 binding pocket of fXa which restrict the preference of this pocket for small and non-polar residues, like Gly [8,29,30]. Consistent with this hypothesis, both fXa cleavage sites on the physiological substrate prothrombin also have a Gly at the P2 positions. It should be noted that a recent kinetic study monitoring the specificity of the cleavage of 10-residue fluorogenic synthetic peptides with an Arg at the P1 position by fXa has identified Phe as the most preferred residue at the P2 position of these substrates [31], suggesting a certain degree of plasticity for the active-site of fXa in interaction with small peptide substrates. Mutants of AT and/or prothrombin with a Phe at the P2 position are required to determine if the results with the small peptide substrates hold true for the macromolecules.

Despite optimal P1 and P2 residues, RCL of AT has been demonstrated to be trapped in a low activity conformation, and thus inhibits fXa slowly (k_2 = 2-3 x 10^3 M^{-1} s^{-1}) unless it binds to heparin-like glycosaminoglycans similar to those found on the surface of endothelium [32]. A full-length heparin accelerates the inhibition of fXa (and other coagulation proteases) by AT by 3 to 4 orders of magnitude, thus the rate of protease inhibition by the serpin is only limited by diffusion [16,33]. This is the basis for the extensive use of heparin for prophylaxis and treatment of venous thrombosis [34]. The dramatic increase in the rate of protease inhibition by AT in the presence of heparin is believed to arise from 1) the ability of heparin to change the conformation of RCL to facilitate its optimal recognition by fXa (activation mechanism) [32,35], and 2) the ability of a full-length heparin to bridge the serpin and enzyme in one complex in the presence of physiological concentrations of Ca^{2+}, promoting the initial interaction between the two proteins (template mechanism) [36]. Thus, the cofactor effect of a full-length heparin accelerates the AT inhibition of fXa ~300-fold through the activation of serpin [35] and ~200-300-fold through a template mechanism in the presence of Ca^{2+} [33]. AT is activated when a unique pentasaccharide sequence of heparin binds to a basic helical structure (helix-D) on AT (Fig. **1**). Structural data have indicated that the P1-Arg (residue 393) of AT is pointing inward and not available for interaction with fXa in the native conformation of the serpin [32,37]. The interaction of the pentasaccharide fragment of heparin with the basic helix-D of AT induces a conformational change in RCL that leads to relocation of P1-Arg from an internal to an external orientation. The conformational change is also linked to the exposure of a cryptic exosite on AT [38,39] that has been demonstrated to be a specific recognition site for interaction with the basic autolysis loop of fXa (residues 143-154) [40,41]. This model of AT interaction with fXa has been supported by the observation that substitution of the autolysis loop of APC, another vitamin K-dependent serine protease completely resistant to inhibition by AT, with the corresponding sequence of fXa renders the mutant APC susceptible to inhibition by AT specifically in the presence of the pentasaccharide fragment of heparin [41]. The mutagenesis study has indicated that Arg-150 of the autolysis loop of fXa is a specific recognition site for interaction with the activated conformation of AT [40].

Unlike the activation mechanism of the serpin, the template effect of heparin in catalyzing the AT inhibition of fXa is a Ca^{2+}-dependent reaction that is mediated through the simultaneous binding of heparin to both AT and fXa (Fig. **1**). The heparin binding site of fXa contains several Arg and Lys residues (Arg-93, Lys-96, Arg-125, Arg-165, Lys-169, Lys-236, and Arg-240), all of which are located in three dimensional regions in the catalytic domain of fXa [8,42] that are also conserved in thrombin [43-46] and fIXa [47]. In the absence of Ca^{2+}, the highly acidic Gla domain of fXa can not fold to its proper conformation, as such binding to the basic exosite of the protease, thereby preventing the protease interaction with heparin. Hence, the template mechanism of heparin in catalyzing the AT inhibition of fXa is only observed in the presence of the metal ion [36]. The same exosite in thrombin is referred to as anion binding exosite-2 [48], and the binding of the AT-heparin complex to this site of thrombin is responsible for the rate accelerating effect of heparin in protease inhibition by AT by a template mechanism [43-45]. In the case of thrombin inhibition, the template effect of heparin is independent of Ca^{2+}, and the protease does not also recognize the activated conformation of the serpin [35,49]. Interestingly, substitution of the autolysis loop of thrombin with the corresponding sequence of fXa enables the mutant protease to specifically react with the activated conformation of AT, further supporting a crucial role for the autolysis loop

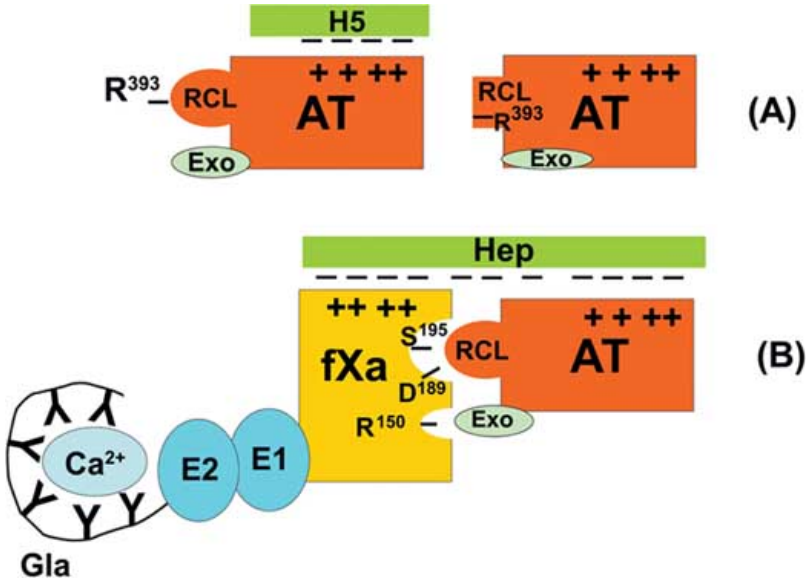

Fig. (1). Model of heparin and pentasaccharide activation of AT and the interaction of the serpin with fXa in the presence of Ca^{2+}. A, the interaction of pentasaccharide (H_5) with a basic region of AT is associated with a conformational change in the reactive center loop (RCL) and the subsequent exposures of P1-Arg and as well as a cryptic exosite (Exo) on the serpin. B, the heparin-activated AT forms a Michaelis-type complex with fXa which is mediated by both active-site dependent interaction of P1-Arg with the primary specificity pocket of fXa (Asp-189) and the exosite-dependent interaction of AT with the autolysis loop of the protease (Arg-150). The complex formation is followed by the catalytic residue Ser-195 attacking P1-Arg of the serpin, thereby initiating the formation of a covalent bond characteristic for the reaction of serine proteases with their substrates during the acylation stage of the reaction. A physiological concentration of Ca^{2+} neutralizes the acidic Gla residues of fXa, thereby enabling a full-length heparin to interact with a cluster of basic residues on the catalytic domain of fXa, thus leading to bridging of the protease and the serpin in one complex and enhancing the reaction by a template mechanism (see text for further discussion).

of fXa in reaction with the activated conformation of the serpin [41]. Taken together, these results suggest that both active-site and heparin-mediated exosite dependent interactions between fXa and AT are required for an effective protease-serpin reaction. It is worth noting that the assembly of fXa into the prothrombinase complex largely protects fXa from inactivation by AT in the presence of heparin [50,51]. This may be due to the occupancy of the AT-heparin interactive sites of fXa by fVa and/or prothrombin in the activation complex [50]. Thus, the requirement for an exosite-dependent interaction mechanism for fXa and the AT-heparin complex likely plays an important physiological role in maintenance of hemostasis.

B. INHIBITION BY ZPI

Similar to AT, ZPI (I04.005) is a serpin with a plasma concentration of 2.6-2.9 μg/ml that interacts with the active-site groove of fXa by a similar covalent mechanism [17,52-54]. However, unlike AT, ZPI is not a universal inhibitor of all coagulation proteases, but instead it is specific for fXa, though recent results have indicated that it also reacts with factors IXa and XIa [53,55]. Moreover, the P1 residue of ZPI is not a typical Arg, but it is a Tyr, and the serpin does not form an SDS-stable complex with fXa, suggesting that, unlike the AT-fXa complex, the ZPI-fXa complex has a higher dissociation constant [17,52,53]. Nevertheless, a covalent interaction of Ser-195 with P1-Tyr of ZPI is required for the recognition mechanism since a 200-fold higher molar concentration of the catalytically inactive Ser-195 to Ala substitution mutant of fXa fails to compete with wild-type protease for interaction with the serpin [54]. Similar to AT, ZPI by itself is a poor inhibitor of fXa, unless it forms a complex with protein Z (PZ) on membrane phospholipids in the presence of Ca^{2+} [17,53]. Unlike the reaction with AT, however, even in complex with PZ, the inhibitory activity of ZPI toward fXa is not complete as measured by an amidolytic activity assay [53,54]. In this assay, ZPI inhibits a maximum of 80% of the protease activity and thereafter no further decline in the amidolytic activity of fXa is observed. The reason for the incomplete inhibitory activity of ZPI toward fXa in the amidolytic activity assays is not known. One possibility is that since ZPI exhibits reversibility in reaction with fXa, the addition of the chromogenic substrate in EDTA (to stop the reaction) and subsequent dilution of the reaction leads to the dissociation of active fXa from the inhibitory complex [53]. Since fXa preparations are a mixture of α and β forms of the protease, the possibility that the two different forms of fXa react differentially with the ZPI-PZ complex, has not been ruled out [53]. In a recent study, under experimental conditions where ~40-60% of the amidolytic activity of fXa was inhibited, second-order rate constants (k_2) of 1.6 x 10^3 M^{-1} s^{-1} in the absence and 6.2 x 10^5 M^{-1} s^{-1} in the presence of PZ were obtained on negatively charged phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine [54]. Thus, the cofactor effect of PZ enhanced the reactivity of ZPI with fXa ~340-fold.

The protein cofactor PZ is a vitamin K-dependent plasma protein with a domain organization identical to that of fXa, and thus capable of interacting with negatively charged

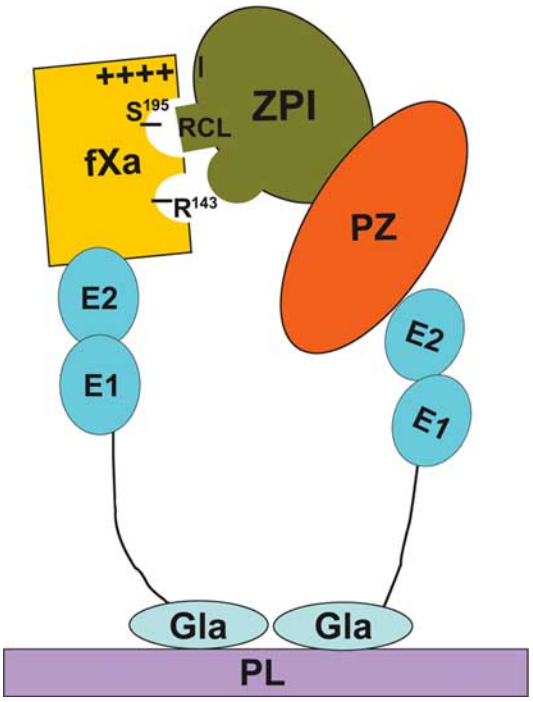

Fig. (2). Model of fXa interaction with the ZPI-PZ complex on the negatively charged membranes. Protein Z (PZ) interaction with a negatively charged membrane surface via the N-terminal Gla-domain condensates ZPI in the vicinity of fXa bound to the same surface. An active-site dependent interaction possibly by a P1-Tyr and Asp-189 (not established experimentally) and an exosite dependent interaction with the autolysis loop of fXa (in particular Arg-143) then leads to the docking of RCL into the active-site pocket of the protease. The complex is further stabilized by additional ionic interactions between basic residues of the heparin-binding exosite (particularly Lys-236) of the protease and a complementary site of the serpin. Similar to AT, the reaction is initiated by Ser-195 forming a covalent bond with the serpin (see text for further discussion). E1, first EGF domain; E2, second EGF domain; PL, phospholipid membrane.

phospholipid membranes via its Gla domain, however, it merely functions as a cofactor in the reaction and lacks any catalytic function [56]. Little is known as to how ZPI interacts with fXa, and unlike the cofactor function of heparin, it is not known how PZ promotes the inhibition of fXa by ZPI on membrane surfaces in the presence of Ca^{2+}. Recent mutagenesis data have indicated that, similar to reaction with AT, both active-site and exosite dependent interaction of ZPI with the same two basic exosites of fXa may be essential for an effective interaction of the serpin with the protease [54]. Nevertheless, instead of Arg-150 of the autolysis loop, which is a recognition site for the protease interaction with the activated conformation of AT [40], Arg-143 of fXa is the crucial recognition site on the protease for interaction with ZPI independent of the cofactor [54]. Heparin plays a minor role on the reactivity of fXa with ZPI [53], however, it appears that ZPI interaction with several heparin-binding exosite residues of fXa including Lys-96, Lys-169, and Lys-236 contributes to the specificity of the serpin-protease interaction [54]. The presence of overlapping binding sites for heparin and ZPI on fXa may provide a possible explanation for the previous observation that the reactivity of the serpin with fXa is not significantly influenced by the polysaccharide [53,54]. Previous results have indicated that certain basic residues of the heparin-binding exosite of fXa also interact with fVa in the prothrombinase complex [42]. It is interesting to note that Arg-165, another heparin-binding residue, is the most important residue on fXa for interaction with fVa in the prothrombinase complex [42], but makes no contact with ZPI since its substitution with Ala has no effect on the reactivity of the mutant with the serpin either in the absence or presence of PZ [54]. Unlike the AT-heparin complex, the assembly of fXa into the prothrombinase complex has little protective effect on the protease inhibition by the ZPI-PZ complex [53]. The lack of requirement for ZPI and PZ interaction with Arg-165 provides a possible explanation for the ability of the serpin to inhibit fXa in the prothrombinase complex [53,54].

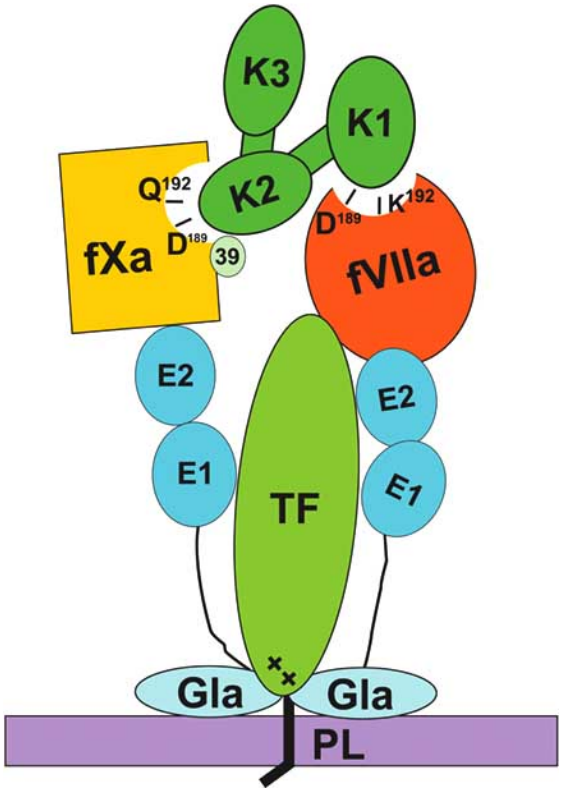

Fig. (3). Model of fXa interaction with TFPI and subsequent inactive quaternary complex formation with fVIIa-TF on the membrane surface. The second Kunitz (K2) domain of TFPI binds to the active-site groove of fXa via P1-Arg (not shown) of the inhibitor interacting with Asp-189 of the protease. This interaction is independent of Ser-195, however, requires Gln-192 for the stability of the binary complex. Extended interactions of TFPI with acidic residues of the 39-loop further stabilize the protease-inhibitor complex. The fXa-TFPI complex then interacts with phospholipid membranes to mediate the binding of the first Kunitz (K1) domain of TFPI to the active-site groove of the fVIIa-TF complex. Similar to interaction with fXa, residues 189 and 192 are required for the effective binding of TFPI to the active-site groove of fVIIa. The recognition of a basic membrane proximal region of TF by the Gla domain of fXa is required for the specificity of this interaction (see text for further discussion).

The mechanism by which the cofactor function of PZ accelerates the reactivity of fXa with ZPI more than 300-fold is not known. In a recent mutagenesis study, a chimeric mutant of fXa containing the Gla domain of APC reacted with the ZPI-PZ complex with ~2-5-fold lower reactivity suggesting that protein-protein interactions via the Gla domains may play some role in the protease recognition by the ZPI-PZ complex [54]. However, an fXa mutant lacking the first EFG domain was inhibited by the serpin-cofactor complex with a normal rate constant [54]. This previous mutagenesis study, with the exception of the Gla domain, did not identify any other interactive site on fXa for PZ [54]. Taken together, it appears that PZ via its Gla domain places ZPI on the negatively charged membrane surface in the vicinity of the active-site groove of fXa, where the protease is bound via its own Gla domain (Fig. **2**). The interaction of the serpin with the autolysis loop of fXa then leads to the docking of RCL into the active-site groove of the protease. The ZPI-PZ complex makes additional stabilizing interactions with the basic residues of the heparin-binding exosite of fXa (in particular with Lys-236) located at the C-terminal helix of the protein [54]. Thus, apart from the active-site, the Gla domain, the basic residues of the autolysis loop (in particular Arg-143) and the heparin-binding exosite of fXa provide most of the binding energy of the protease interaction with the ZPI-PZ complex.

C. INHIBITION BY TFPI

Unlike the two serpin inhibitors of fXa discussed above, TFPI (LI02-002) is a slow and tight-binding, Kunitz-type inhibitor that is capable of rapidly inhibiting fXa independent of a cofactor [18,57,58]. TFPI has three Kunitz-type domains. It regulates coagulation by the fXa-dependent inhibition of the fVIIa-tissue factor (TF) complex during the initial phase of the clotting cascade [18]. It functions by first binding to the active-site groove of fXa by the second Kunitz domain, and thereafter binding tightly to the active-site of the fVIIa-TF complex on membrane surfaces via the first Kunitz domain [18,57], thereby rendering both proteases inactive in a quaternary complex (Fig. **3**). It has been demonstrated that the interaction of the Gla domain of fXa with a basic membrane proximal region of TF is required for the specificity of this interaction [59]. The function of the third Kunitz domain of TFPI is not well characterized, though results of a recent mutagenesis study indicated that a possible role for this domain may involve the cell surface localization of the inhibitor [60]. Similar to both of the serpin inhibitors, TFPI makes extensive interactions with both the active-site, and exosites remote from the active-site groove of fXa in order to rapidly inhibit the protease [61]. Unlike both serpins, however, no cofactor is required for the high affinity interaction of TFPI with fXa (K_i < 0.1 nM). On the other hand, the TFPI inhibition of the fVIIa-TF complex requires prior complex formation of the inhibitor with fXa on the membrane surface. It should be noted that heparin is also known to enhance the inhibitory effect of TFPI toward fXa [62], possibly by binding to a stretch of basic residues at the C-terminus of the inhibitor. It has been demonstrated that the binding of the C-terminal basic residues of TFPI to heparan sulfate proteoglycans on cell surfaces also facilitates the uptake and degradation of TFPI-fXa complexes [63]. Nevertheless, the specific receptor responsible for the TFPI-fXa complex degradation has not been identified, though it has been demonstrated that this receptor is neither LRP nor TF [64]. Similar to protection from inhibition by AT, fXa in the prothrombinase complex is also resistant to inhibition by physiological concentrations of TFPI (< 8 nM) in the presence of its physiological substrate, prothrombin in either the absence or presence of heparin [62].

In contrast to a longer, flexible and disordered conformation for RCL in AT, TFPI has a shorter, fixed and canonical RCL that undergoes minimal changes in the structure upon complex formation with the protease [61]. In this mechanism of interaction referred to as a "lock and key" type of interaction [65], the binding is characterized by a thermodynamic stabilization in which all binding energy is utilized to stabilize a reversible Michaelis-type complex [65,66]. Thus, unlike the kinetically stable and irreversible fXa-AT complex, the fXa-TFPI complex is reversible and a high-affinity complex formation is independent of the catalytic residue Ser-195 [54]. This is evidenced by the observation that the inactive Ser-195 to Ala substitution mutant of fXa can effectively compete with the wild-type fXa for high affinity binding to TFPI [54]. Unlike Ser-195, previous mutagenesis studies have indicated that Gln-192 of fXa is essential for the stability and high affinity interaction of the protease with TFPI [67]. The nature of residue 192 in the active-site groove is a critical factor in determining the specificity of coagulation proteases in interaction with TFPI. Thus, fXa which has a Gln at this position can effectively bind to the inhibitor [67]. On the other hand, the presence of a Glu at the corresponding position of both thrombin and APC prevents the interaction of both proteases with TFPI [67]. In support of this hypothesis, the substitution of Glu-192 with a Gln in both thrombin and APC improves the reactivity of these proteases with TFPI by three orders of magnitude [67]. The three dimensional structure of the isolated Kunitz-2 domain of TFPI in complex with porcine trypsin has been resolved [61]. It has been noted that the carboxamide nitrogen atom of Gln-192 hydrogen bonds to backbone carbonyl groups of Cys-14 and Ile-13 at the P2 and P3 sites of TFPI, respectively [61]. Structural data suggests that Gln-192 of trypsin also makes similar hydrogen-bonds with backbone carbonyl groups of pancreatic trypsin inhibitor (BPTI) to stabilize the binary complex [68]. Thus, Gln-192 of fXa, most likely, makes similar hydrogen-bonds with backbone carbonyl groups of the second Kunitz domain of TFPI, explaining the key role of this residue in the interaction. Residue 192, which is a Lys in fVIIa, is also critical for determination of the binding specificity of the first Kunitz domain of TFPI since the substitution of Lys-192 with either Gln or Glu abolishes the ability of the mutant proteases to interact with the inhibitor [69]. The nature of interaction of Lys-192 of fVIIa with the first Kunitz domain of TFPI has not been studied.

Unlike the important role of the P2-binding residue Tyr-99 in fXa reaction with AT, this residue makes a minimal contribution to specificity of the protease interaction with TFPI [29]. Molecular modeling, based on the x-ray crystal structure of the second Kunitz domain of TFPI in complex with trypsin, has predicted that the interaction of basic residues of the autolysis loop of fXa with an acidic patch of

TFPI likely contributes to the specificity of the interaction [61]. However, single Ala substitution mutants of basic residues of the autolysis loop (Arg-143, Lys-147, Arg-150, and Arg-154) did not alter the equilibrium dissociation constants (K_i) for the interaction of the fXa mutants with TFPI more than 2-fold [40]. These results do not support a dominant role for the autolysis loop of fXa in specificity of interaction with the inhibitor. On the other hand, the substitution of the acidic Glu residues of the 39-loop (Glu-36, Glu-37, and Glu-39) with Ala leads to a significant impairment in the ability of the mutants to interact with TFPI, suggesting a role for this loop in the protease-inhibitor interaction [70]. This is consistent with the structural data [61]. Previous mutagenesis data has also implicated an important role for the basic residues of the Na^+-binding 225-loop (Arg-221, Lys-222 and Lys-224) in fXa interaction with both TFPI and AT [11]. Unfortunately, however, the mutagenesis of this loop also dramatically impairs the amidolytic and proteolytic activities as well as the Na^+-binding affinity of the mutant proteases, thus complicating the interpretation of the kinetic data [11].

In addition to TFPI, a number of non-physiological, but naturally occurring peptide inhibitors specific for fXa have recently been identified which inhibit the protease by a similar slow and tight-binding inhibition mechanism [71-74]. The interaction of a recombinant form of such an inhibitor possessing potent antithrombotic properties, derived from the soft tick *Ornithodoros moubata* (tick anticoagulant peptide, TAP, I52.001), with fXa has been extensively studied [72]. The kinetic data have indicated that recombinant TAP (rTAP) inhibits fXa with an equilibrium dissociation constant of ~0.2 nM [72]. Mutagenesis data have indicated that both active-site and exosite dependent interaction of rTAP with fXa is also required for the characteristic slow and tight-binding inhibition mechanism [75]. Interestingly, unlike TFPI, residue 192 does not contribute to the high affinity interaction of fXa with rTAP, however, similar to reactions with the serpins, both of the P2-binding residues Tyr-99 and Phe-174 and the autolysis loop (Arg-143) are essential for the interaction [75]. Both of the P2-binding residues also make a dominant contribution to the binding energy of fXa interaction with the synthetic small molecular weight inhibitor (2S)-2-[4-[[(3S)-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-(7-aminido-2-naphthyl)propanic acid hydrochloride pentahydrate (DX-9065a) [76] developed as a specific fXa inhibitor by Daiichi Pharmaceuticals Co. in Japan. For detailed information relating to the mechanism of inhibition of fXa by both synthetic and natural non-physiological, but therapeutically relevant inhibitors see Refs. [72,73,77-80].

In summary, fXa interaction with both of its physiological serpin inhibitors requires Asp-189 and Ser-195 for covalent interaction with the P1 residues of inhibitors. In reaction with AT, the interaction of the aryl-binding pocket residues Tyr-99 and Phe-174 with the P2-Gly of the serpin further contributes to the affinity of the interaction. The role of the P2-binding pocket of fXa in reaction with ZPI has not been studied. Heparin-mediated interaction of AT with both the autolysis loop (Arg-150) and the heparin-binding exosite of fXa is required for an effective inhibition of the protease by the serpin. In the case of ZPI, the interaction of the Gla domains of fXa and PZ on negatively charged membrane surfaces and the interaction of ZPI with the autolysis loop (Arg-143) and selected heparin-binding residues of fXa (particularly Lys-236) account for the most of the binding energy of the interaction. In the case of TFPI, the catalytic residue Ser-195 plays a minimal role, however, Gln-192 plays a decisive role in the inhibitor recognition mechanism. The exosite dependent interaction of TFPI with fXa appears to be mediated through the specific interaction of the inhibitor with an acidic region of the 39-loop and possibly also the basic residues of the Na^+-binding 225-loop. The autolysis loop residues of fXa play a less critical role in interaction with this inhibitor. Noting that both intrinsic and extrinsic pathways of coagulation converge at the point of fXa generation, fXa is an ideal target for anticoagulant drugs. Thus, identification of the molecular determinants of the active-site and exosite binding specificity of fXa as discussed in this review paves the way for rational design of novel fXa inhibitors potentially useful for management and treatment of thrombosis.

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