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Structure of human factor VIIa/tissue factor in complex with a peptide-mimetic inhibitor: high selectivity against thrombin by introducing two charged groups in P2 and P4

Shojiro Kadono,* Akihisa Sakamoto, Yasufumi Kikuchi, Masayoshi Oh-eda, Naohiro Yabuta, Takaki Koga, Kunihiro Hattori, Takuya Shiraishi, Masayuki Haramura, Hirofumi Kodama, Yoshiyuki Ono, Toru Esaki, Haruhiko Sato, Yoshiaki Watanabe, Susumu Itoh, Masateru Ohta and Toshiro Kozono

Fuji Gotemba Research Laboratories, Chugai Pharmaceutical Co. Ltd, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan

Correspondence e-mail: kadonosuj@chugai-pharm.co.jp

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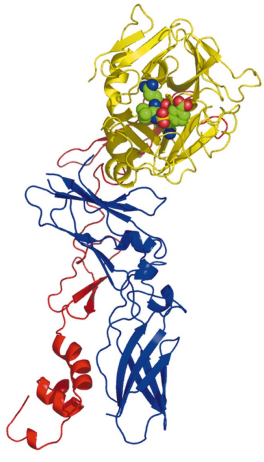


The crystal structure of human factor VIIa/soluble tissue factor (FVIIa/sTF) in complex with a highly selective peptide-mimetic FVIIa inhibitor which shows 1670-fold selectivity against thrombin inhibition has been solved at 2.6 Å resolution. The inhibitor is bound to FVIIa/sTF at the S1, S2 and S3 sites and at the additional S1 subsite. Two charged groups, the amidino group in P2 and the carboxylate group in P4, form ionic interactions with Asp60 and Lys192 of FVIIa, respectively. Structural comparisons between factor VIIa and thrombin show that thrombin has oppositely charged residues, Lys60F and Glu192, in the S2 site and the S1 subsites, respectively. These data suggest that the utilization of the differences of charge distribution in the S2 site and the S1 subsites between FVIIa and thrombin is critical for achieving high selectivity against thrombin inhibition. These results will provide valuable information for the structure-based drug design of specific inhibitors for FVIIa/TF.

1. Introduction

Coagulation factor VIIa (FVIIa; EC 3.4.21.21) is a serine protease enzyme which plays an important role in blood coagulation. The blood-coagulation cascade is divided into extrinsic and intrinsic coagulation pathways. FVIIa in complex with tissue factor (TF) initiates the extrinsic coagulation pathway. This complex activates factors IX to IXa and X to Xa, which in turn activate factor X to Xa and prothrombin to thrombin, respectively (Davie *et al.*, 1991). Thrombin cleaves fibrinogen to fibrin, which forms blood clots with activated platelets. Inappropriate thrombus formation in blood vessels causes cardiovascular diseases (myocardial infarction, stroke, pulmonary embolism *etc.*), which are the most common causes of mortality in the industrialized world (Braunwald *et al.*, 2000). Recent studies on blood coagulation have suggested that selective inhibition of extrinsic coagulation provides effective anticoagulation and low risk of bleeding compared with other antithrombotic mechanisms (Harker *et al.*, 1996; Himber *et al.*, 1997; Szalony *et al.*, 2002). Thus, specific FVIIa/TF complex inhibition, which blocks only extrinsic coagulation, is seen as a promising target for developing new anti-coagulant drugs (Klingler *et al.*, 2003; Parlow *et al.*, 2003).

Compound (1) is a potent and highly selective thrombin peptide-mimetic inhibitor (Wiley *et al.*, 1996) which showed micromolar inhibition activity for human FVIIa/TF in our assay (Fig. 1). Based on the structural information of compound (1) bound to thrombin (Wiley *et al.*, 1996) and of covalently inhibited FVIIa/TF (Banner *et al.*, 1996), the addition of a substituted sulfonamide moiety at the N-terminus (P4) and the modification of the P2 and P3 moieties was carried out to improve inhibition for FVIIa/TF and selectivity against thrombin inhibition. In this study, the ratio of IC_{50} for thrombin divided by the IC_{50} for FVIIa/TF is used as an indicator of selectivity against thrombin inhibition. A large value of the IC_{50} ratio shows high selectivity against thrombin inhibition. Thrombin inhibition is thought likely to be a crucial factor in bleeding risk since thrombin cleaves fibrinogen to fibrin as a final step of blood coagulation. Therefore, high selectivity against thrombin inhibition is thought to be important. As a result of optimization of compound (1), we reported a compound (1) derivative, propylsulfonamide-D-Thr-Met-*p*-aminobenzamidine, that showed submicromolar inhibition activity for FVIIa/TF and 26-fold selectivity against thrombin inhibition; its

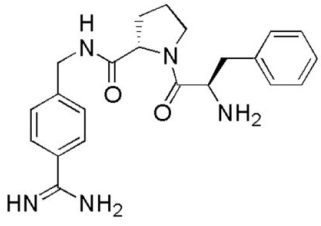
binding mode in FVIIa and the soluble domain of tissue factor (sTF) complex was revealed by X-ray crystallography (Kadono *et al.*, 2004). This compound achieved a remarkable improvement (26 000-fold) in selectivity against thrombin inhibition over compound (1). However, this compound has insufficient selectivity against thrombin inhibition. Our continual efforts to modify the peptide-mimetic inhibitor resulted in a novel peptide-mimetic FVIIa inhibitor with two charged groups, the 4-amidino-2-aminobutylic acid moiety in P2 and the *meta*-carboxylic benzylsulfonamide moiety in P4 (Fig. 1, compound 2). Compound (2) showed potent inhibition activity for FVIIa/TF (IC_{50} = 17 n*M*) and exhibited high selectivity against thrombin inhibition (1670-fold). On the other hand, compound (3) (Fig. 1), which has no charged group in P2 and P4, exhibited low selectivity against thrombin inhibition (sixfold). The introduction of two charged groups in P2 and P4 leads to a 280-fold improvement of selectivity against thrombin inhibition. Thus, these charged groups are critical for achieving high selectivity against thrombin inhibition.

In this study, we determined the X-ray crystal structure of compound (2) bound to FVIIa/sTF in order to reveal the interactions between FVIIa/sTF and the inhibitor. Based on this structure, key interactions for achieving high selectivity against thrombin inhibition will be discussed.

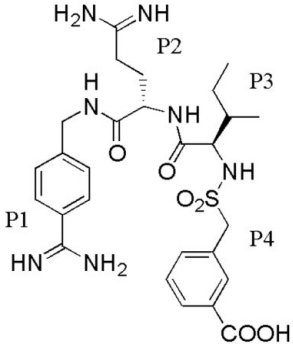
2. Materials and methods

2.1. Compound synthesis and inhibition assay

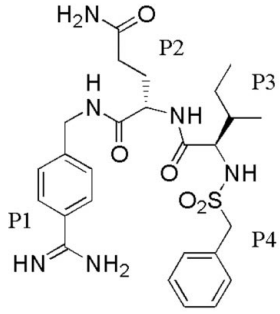
The peptide-mimetic FVIIa/TF inhibitors used in this study were synthesized in our laboratory. The detail of the synthesis and structure–activity relationships will be reported elsewhere. Inhibition activities for human FVIIa/TF and thrombin were measured using chromogenic substrates as described previously (Kadono *et al.*, 2004).



Compound (1)
FVIIa IC_{50} (n*M*) 2200
Thrombin IC_{50} (n*M*) 2.2 (0.001)



Compound (2)
FVIIa IC_{50} (n*M*) 17
Thrombin IC_{50} (n*M*) 28 300 (1670)



Compound (3)
FVIIa IC_{50} (n*M*) 25
Thrombin IC_{50} (n*M*) 150 (6)

Figure 1
Chemical structures of peptide-mimetic thrombin and factor VIIa inhibitors. Values in parentheses refer to the ratio against FVIIa/TF IC_{50}.

Table 1
X-ray diffraction data-collection and refinement statistics.

Values in parentheses are for the outer resolution shell.

Data collection	
Wavelength (Å)	1.00
Space group	$P2_12_12_1$
Unit-cell parameters	
a (Å)	71.56
b (Å)	82.30
c (Å)	123.52
Resolution (Å)	50.0–2.5 (2.64–2.5)
Total reflections	110911
Unique reflections	25939
Completeness (%)	100.0 (100.0)
R_{merge} (%)	10.6 (34.3)
$I/\sigma(I)$	7.9
Refinement	
Resolution (Å)	20.0–2.6
Reflections used	23023
R_{cryst} (%)	19.3
R_{free} (%)	25.0
R.m.s.d. bonds (Å)	0.007
R.m.s.d. angle (°)	1.4
No. of non-H atoms	
Protein	4697
Inhibitor	41
Water molecules	294
Ca^{2+}	9

2.2. Crystallization

Human FVIIa/sTF was prepared as described previously (Kadono *et al.*, 2004; Kirchhofer *et al.*, 1995). Crystals of the FVIIa/sTF–compound (2) complex were obtained by the hanging-drop vapour-diffusion method at 298 K using protein at a concentration of 12 mg ml^{-1}, 50 m*M* Tris–HCl pH 7.5, 5 m*M* $CaCl_2$, 100 m*M* NaCl and 0.5 m*M* compound (2). The protein solution and a reservoir solution consisting of 6–8% PEG 5000, 100 m*M* cacodylate buffer pH 5.0 were mixed with microseed crystals of FVIIa/sTF covalently inhibited by D-Phe-Phe-Arg chloromethyl ketone which were prepared as described previously (Kadono *et al.*, 2004; Kirchhofer *et al.*, 1995).

2.3. Data collection and refinement

After soaking in a cryoprotectant solution of 10% PEG 5000, 100 m*M* cacodylate buffer pH 5.0, 100 m*M* NaCl, 5 m*M* $CaCl_2$,

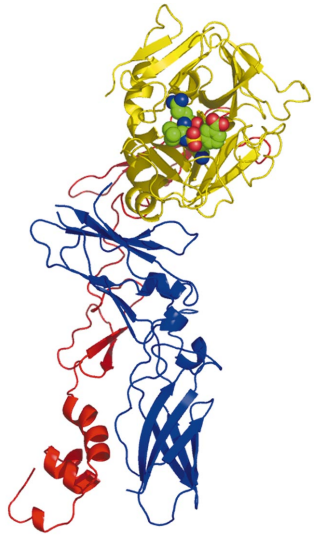


Figure 2
The overall fold of FVIIa/sTF in complex with compound (2). The heavy chain of FVIIa is shown in yellow. The light chain of FVIIa is shown in red. The soluble domain of TF is shown in blue. C atoms of compound (2) (space-filled model) are shown in green.

30%(*v*/*v*) glycerol, the crystals were frozen at 100 K in a nitrogen-gas stream. X-ray diffraction data from the FVIIa/sTF crystal in complex with compound (2) were collected at beamline BL-40B2 of SPring-8. The data were processed using *MOSFLM* (Leslie, 2003) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

The crystal of FVIIa/sTF in complex with compound (2) is isomorphous with the crystal of FVIIa/sTF in complex with D-Phe-Phe-Arg chloromethyl ketone (Kirchhofer *et al.*, 1995). Therefore, the model phases of FVIIa/sTF bound to compound (2) were improved by rigid-body refinement using the previously published structure of FVIIa/sTF in complex with D-Phe-Phe-Arg chloromethyl ketone (PDB code 1dan; Banner *et al.*, 1996) as a starting model. After a rigid-body refinement, the compound (2) molecule was placed in the electron density using a Fourier difference map. The model was refined by iterative cycles of *B*-factor and positional refinement followed by simulated annealing with slow-cooling protocols using the *CNX* package (Accelrys). Gradually, water molecules were inserted during refinement. A randomly selected 7% of the data set was used for $R_{\rm free}$ calculation. Model building of the protein, inhibitor and water molecules was performed with *QUANTA* (Accelrys). X-ray diffraction data-collection and refinement statistics are shown in Table 1. The Ramachandran conformational parameters from the last cycle of refinement generated by *PROCHECK* (Laskowski *et al.*, 1993) show that 84.9% of the residues have the most favoured conformation.

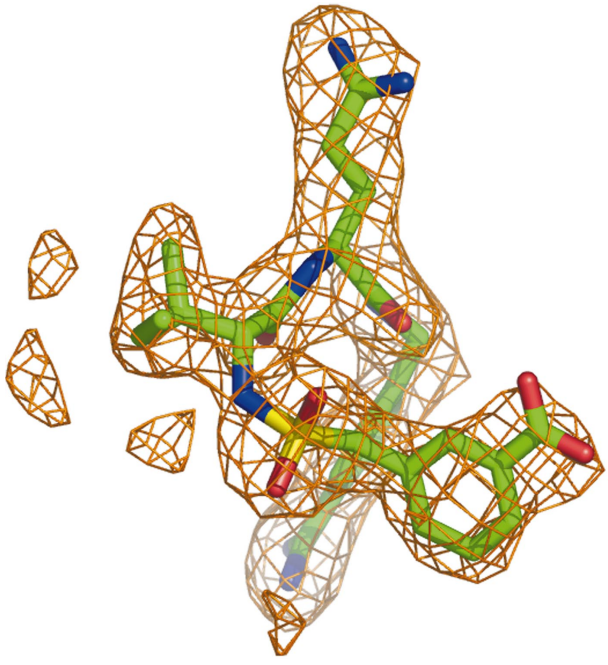


Figure 3
$F_o - F_c$ omit map of compound (2) contoured at 2.25σ. C atoms of compound (2) are shown in green.

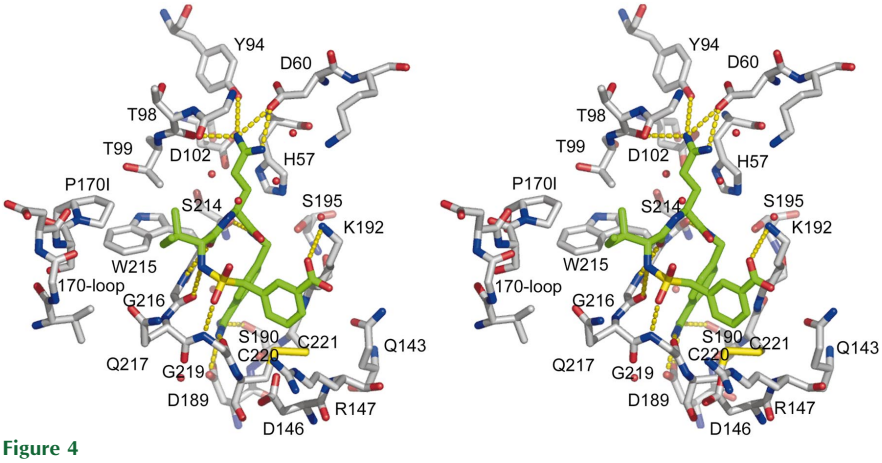


Figure 4
X-ray crystal structure of compound (2) bound to factor VIIa/sTF. C atoms of compound (2) are shown in green.

3. Results and discussion

FVIIa consists of two chains: the heavy chain and the light chain. The two chains are linked *via* a disulfide bridge. The heavy chain is an arginine-specific serine protease of the thrombin/trypsin family (Banner *et al.*, 1996). Compound (2) is bound to the heavy chain of FVIIa/sTF (Fig. 2) at the S1, S2 and S3 sites and at the additional site that consists of a Cys191–Cys220 disulfide bridge, Gln143, Asp146, Gly219 and Lys192 (the S1 subsite) similar to the binding of propylsulfonamide-D-Thr-Met-*p*-aminobenzamidine reported previously (Kadono *et al.*, 2004). The numbering of the FVIIa and thrombin residues used is based on topological equivalences with chymotrypsin (Banner *et al.*, 1996; Bode *et al.*, 1989). The final electron density of compound (2) is well defined (Fig. 3). The detailed active-site structure of FVIIa/TF in complex with compound (2) is shown in Fig. 4. The amidino group of the benzamidine moiety forms a symmetric salt bridge with the side-chain carboxylate of Asp189 and is stabilized further by hydrogen bonds with the main chain of Gly219 and the side chain of Ser190 in the S1 site of FVIIa/sTF. The peptidyl chain atoms of compound (2) make hydrogen bonds with the main chain of Ser214 and Gly216, forming a short antiparallel β-sheet. The side chain of Asp60 makes a hydrophilic pocket with the side-chain O atom of Tyr94 and the main-chain O atom of Thr98 in the S2 site of FVIIa/sTF. This pocket is a unique feature of the active site of FVIIa/sTF. The amidino group in P2 of compound (2) occupies this hydrophilic pocket. The amidino group in P2 makes good ionic interactions with Asp60 and in addition makes hydrogen bonds with the side-chain O atom of Tyr94 and the main-chain O atom of Thr98. The distances between the N atoms of the amidino group in P2 and the nearest carboxylate O atom of Asp60 are 2.8 and 2.9 Å, respectively. A pocket in the S2 site of FVIIa/sTF consisting of Thr99, Asp102 and Ser214 is occupied by a water molecule, which makes a hydrogen bond with the side-chain carboxylate of Asp102 and a van der Waals contact with the P2 moiety of compound (2). The S3 site is occupied by the D-isoleucine side chain in P3 of compound (2), which fits the small S3 site of FVIIa/sTF well and makes van der Waals contacts with Thr99, Pro170I and Trp215. In particular, the D-isoleucine side chain in P3 makes good hydrophobic interactions with Pro170I. Next to the S3 site, there is weak electron density for residues 170D–170H in the 170-loop. The *meta*-carboxylic benzylsulfonamide moiety in P4 of compound (2) occupies the S1 subsite of FVIIa/sTF. One of the sulfonamide O atoms in P4 makes a hydrogen bond with the main-chain N atom of Gly219. The benzyl group in P4 fits the S1 subsite well and makes van der Waals

contacts with Asp146, Lys192, Gly219 and Cys220. In particular, the methylenes of the Lys192 side chain make good hydrophobic interactions with the benzene moiety. In addition, the carboxylate group makes an ionic interaction with Lys192. The shortest distance between the side-chain N atom of Lys192 and the O atoms of carboxylate group of compound (2) is 3.8 Å. Consequently, compound (2) shows electrostatic complementarity at the S1 and S2 sites, and the S1 subsite of FVIIa/sTF (Fig. 5).

Two charged groups, the amidino group in P2 and the carboxylate group on the benzylsulfonamide moiety in P4, make ionic interactions with Asp60 and Lys192 of FVIIa/sTF, respectively. However, the inhibition activity for FVIIa/TF of compound (2) changes little compared with compound (3), which should have no ionic interaction with Asp60 and Lys192 of FVIIa/sTF. In general, the contribution of ionic interactions to ligand-binding free energy is not significant in the solvent-exposed region because of the large free-energy penalty of desolvation of charged groups (Froloff *et al.*, 1997; Hendsch & Tidor, 1994). The side chain of Lys192 of FVIIa/sTF is fully exposed to solvent and the side chain of Asp60 of FVIIa/sTF is near the solvent region and partially exposed to solvent. Therefore, the introduction of two charged groups would not cause an increase of inhibition activity for FVIIa/TF. Hydrophobic interactions made by the D-isoleucine side chain in P3 and the benzyl group in P4 may be responsible for the potent inhibition of FVIIa/TF by compound (2).

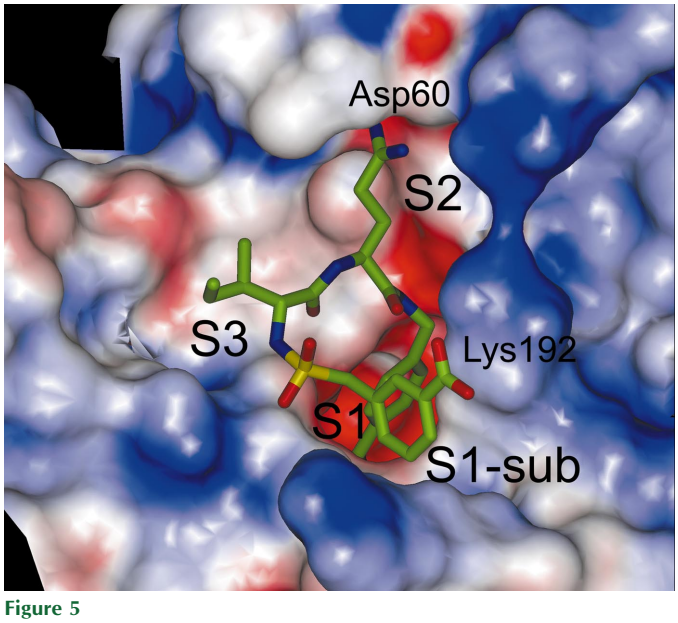


Figure 5
Electrostatic surface potentials at the active site of FVIIa/sTF bound to compound (2). The surface colours represent the potential, ranging from $-6kT$ (red) to $+6kT$ (blue). C atoms of compound (2) (stick model) are shown in green. The electrostatic potentials were calculated with *DELPHI* (Accelrys). The surface was drawn with *INSIGHT II* (Accelrys).

On the other hand, the introduction of two charged groups in P2 and P4 led to a large improvement of selectivity against thrombin inhibition. The structure of compound (1) bound to thrombin has been solved by X-ray crystallographic analysis (Wiley *et al.*, 1996), but the structure is not publicly available in the Protein Data Bank (Berman *et al.*, 2000). Therefore, the structure of compound (2) bound to FVIIa/sTF was compared with the structure of D-Phe-Pro-Arg chloromethyl ketone (PPACK) bound to thrombin (Bode *et al.*, 1989), which has a chemical structure similar to that of compound (1). FVIIa and thrombin belong to the same serine protease family and their catalytic domains (or heavy chains) have similar folds. However, structural variability is found in several regions (Fig. 6). At the S2 site, thrombin has a lid that is formed by the hydrophobic residues Tyr60A and Trp60D (Banner & Hadvary, 1991; Bode *et al.*, 1989). The S2 site of thrombin fits to the small and hydrophobic proline moiety in P2. Thrombin has a large and hydrophobic S3 site consisting of Leu99, Ile174 and Trp215, which fits the large D-phenylalanine moiety in P3 of PPACK (Banner & Hadvary, 1991; Bode *et al.*, 1989). Therefore, the introduction of the relatively large P2 moiety of compound (2) seems to cause steric repulsion. The introduction of the relatively small D-isoleucine side chain in P3 of compound (2) would create a vacancy in the large S3 site of thrombin and lead to a relative reduction of the binding affinity for thrombin. In addition, thrombin has a positively charged residue, Lys60F, in the S2 site (Banner & Hadvary, 1991; Bode *et al.*, 1989). The modification from the amide group in P2 to the positively charged amidino group would cause an electrostatic repulsion with the Lys60F of thrombin (Fig. 6). Furthermore, thrombin has a negatively charged residue, Glu192 (Banner & Hadvary, 1991; Bode *et al.*, 1989), in the S1 subsite instead of Lys192 of FVIIa. Glu192 of thrombin would cause an electrostatic repulsion with the negatively charged carboxylate group on the benzylsulfonamide moiety in P4 (Fig. 6). These electrostatic repulsions with Lys60F and Glu192 would result in a large reduction of inhibition activity for thrombin. A combination of the electrostatic attractions with Asp60 and Lys192 of FVIIa and the electrostatic repulsions with Lys60 and Glu192 of thrombin would lead to a high selectivity of compound (2) against thrombin inhibition.

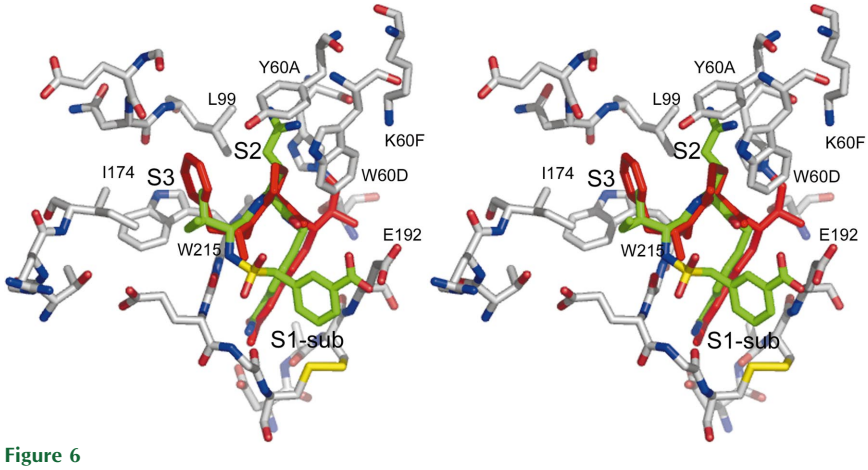


Figure 6
Superposition of the coordinates of compound (2) from the FVIIa/sTF–compound (2) complex on the coordinates of the active site of thrombin from the thrombin–D-Phe-Pro-Arg chloromethyl ketone complex (PDB code 1ppb). C atoms of compound (2) are shown in green. Atoms of D-Phe-Pro-Arg chloromethyl ketone are show in red. Figs. 2–4 and 6 were created using *PyMol* (DeLano Scientific, http://www.pymol.org).

In summary, this crystal structure revealed that Asp60 in the S2 site of FVIIa/TF and Lys192 in the S1 subsite of FVIIa/TF are key residues for achieving high (1670-fold) selectivity against thrombin inhibition. This high selectivity against thrombin inhibition is probably a consequence of the differences in the charged residues in the S2 site and the S1 subsite between FVIIa and thrombin. These results will provide valuable information for achieving greater selectivity against thrombin inhibition and for the rational and rapid development of specific inhibitors for FVIIa/TF.

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