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Identification of the site of binding of sulfated, low molecular weight lignins on thrombin

May H. Abdel Aziz, Philip D. Mosier, and Umesh R. Desai*

Department of Medicinal Chemistry and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, VA 23298

Abstract

Sulfated, low molecular weight lignins (LMWLs), designed recently as macromolecular mimetics of the low molecular weight heparins (LMWHs), were found to exhibit a novel allosteric mechanism of inhibition of human thrombin, factor Xa and plasmin, which translates into potent human blood anticoagulation potential. To identify the site of binding of sulfated LMWLs, a panel of site-directed thrombin mutants was studied. Substitution of alanine for Arg⁹³ or Arg¹⁷⁵ induced a 7–8-fold decrease in inhibition potency, while Arg¹⁶⁵Ala, Lys¹⁶⁹Ala, Arg¹⁷³Ala and Arg²³³Ala thrombin mutants displayed a 2–4-fold decrease. Other exosite 2 residues including those that play an important role in heparin binding, such as Arg¹⁰¹, Lys²³⁵, Lys²³⁶ and Lys²⁴⁰, did not induce any deficiency in sulfated LMWL activity. Thrombin mutants with multiple alanine substitution of basic residues showed a progressively greater defect in inhibition potency. Comparison of thrombin, factor Xa, factor IXa and factor VIIa primary sequences reiterated Arg⁹³ and Arg¹⁷⁵ as residues likely to be targeted by sulfated LMWLs. The identification of a novel site on thrombin with capability of allosteric modulation is expected to greatly assist the design of new regulators based on the sulfated LMWL scaffold.

1. Introduction¹

Allosteric regulation of coagulation enzymes, especially thrombin and factors Xa, IXa, and XIa, is a fundamental property exploited by nature [1,2] to maintain homeostatic balance between coagulation and anticoagulation. The primary allosteric regulator of these enzymes is heparin, an animal-derived mixture of millions of polysaccharide chains, which binds in a site remote from the active site and enhances the inhibition of the enzymes by antithrombin, a plasma glycoprotein inhibitor [3].

Although heparins (unfractionated heparin or low molecular weight heparin (LMWH)) have been used as anticoagulants since a long time, the agents are beset with a number of adverse reactions including enhanced bleeding risk, immunological reaction, poor oral bioavailability, patient-to-patient response variability, narrow therapeutic index, possibility of contamination and others [2,4,5]. Yet, it has been difficult to replace these macromolecular entities because these are excellent anticoagulants and fairly inexpensive.

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*Address for correspondence: Department of Medicinal Chemistry, Virginia Commonwealth University, 800 E. Leigh St., Suite 212, PO Box 980133, Richmond, VA 23219. Ph. 804-828-7328, Fax 804-827-3664, urdesai@vcu.edu.

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We recently designed sulfated LMWLs as macromolecular mimetics of LMWHs that exhibited potent inhibition of coagulation *in vitro* and *ex vivo* [6-8]. Sulfated LMWLs are sulfated oligomers of varying lengths and substitution pattern (Fig. 1) that attempt to mimic the structural diversity of LMWHs and are readily synthesized in a simple two-step chemical process. Structurally, sulfated LMWLs possess an aromatic backbone decorated with few sulfate and carboxylate groups, while LMWHs are highly anionic, carbohydrate-based molecules. The combination of a hydrophobic, aromatic scaffold and selected number of charged groups in sulfated LMWLs induces novel physicochemical and protein recognition properties [7-9]. In this respect, sulfated LMWLs are proving to be unlike any other class of anticoagulants being investigated to-date, including the heparins, the coumarins, the hirudins, the peptidomimetics and the small molecule direct inhibitors [2,10].

Functionally, sulfated LMWLs display plasma and blood anticoagulation profiles similar to that of LMWHs [8]. Enzyme inhibition studies have shown that these molecules inhibit thrombin, factor Xa and plasmin in an antithrombin-independent manner [6,11]. Interestingly, mechanistic studies have shown that sulfated LMWLs utilize exosite 2 of thrombin to induce inhibition [7]. This novel allosteric inhibition mechanism distinguishes sulfated LMWLs from the heparins, which do not directly inhibit these enzymes. In fact, sulfated LMWLs appear to be the only molecules that allosterically induce inhibition of thrombin through an exclusive exosite 2 interaction.

To elucidate the site of binding of sulfated LMWLs, we studied the inhibition properties of a panel of single, double and triple site-directed thrombin mutants. The results show that Arg⁹³ and Arg¹⁷⁵ are two key residues that recognize CDSO3, a specific, highly potent sulfated LMWL, while Arg¹⁶⁵ and others are also important. Comparison of thrombin, factor Xa, factor IXa and factor VIIa primary sequences supports these conclusions. The identification of a novel site on thrombin with capability of allosteric modulation is expected to greatly assist the design of new regulators based on the sulfated LMWL scaffold.

2. Materials and Methods

2.1 Sulfated LMWL, Chromogenic Substrate and Recombinant Thrombins

CDSO3, a specific sulfated LMWL (Fig. 1), was synthesized in two steps from caffeic acid using chemo-enzymatic synthesis described by Monien *et al* [6]. The average molecular weight of CDSO3 was measured using a Shodex Asahipak GS-320 HQ size-exclusion (SEC) column (Showa Denko America, Inc, New York, NY) eluted with 0.1 M NaOH at 0.7 mL/min and detected at 280 nm. Polystyrene sulfonate (PSS) standards (4200–33000) from American Polymer Standards (Mentor, OH) were used as standards for calibration of the column. The average molecular weight of CDSO3 was calculated to be 3,320 Da. Chromogenic substrate S2238 (H-*D*-Phe-Pip-Arg-*p*-nitroanilide) was purchased from ANASPEC (Fremont, CA). All other chemicals were analytical reagent grade from either Sigma Chemicals (St. Louis, MO) or Fisher (Pittsburgh, PA) and used as such.

Recombinant wild-type and mutant thrombins were prepared in the Rezaie laboratory at St. Louis University School of Medicine, as described earlier [12,13]. Briefly, Arg⁹³Ala, Arg⁹⁷Ala, Arg¹⁰¹Ala, Arg¹⁶⁵Ala, Lys¹⁶⁹Ala, Arg¹⁷³Ala, Arg¹⁷⁵Ala, Arg²³³Ala, Lys²³⁵Ala, Lys²³⁶Ala, or Lys²⁴⁰Ala thrombin was prepared in prothrombin-1 form by PCR mutagenesis and expression in baby hamster kidney cells (BHK) using the pNUT-PL2 expression/purification vector system. The mutants were purified to homogeneity by immunoaffinity chromatography using the Ca²⁺-dependent monoclonal antibody, HPC4, and activated to thrombin. The active-site concentrations of thrombin mutants were determined by an amidolytic activity assay and stoichiometric titrations with antithrombin [12,13]. These concentrations were within 90-100% of those expected on the basis of their

absorbance at 280 nm. Stock solutions of these thrombins were prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl and 2.5 mM CaCl₂.

2.2 Quantitative Measurement of Thrombin Inhibition Potential of CDSO3

CDSO3 inhibition of recombinant wild-type and mutant thrombins was studied in a manner identical to that described earlier for human plasma thrombin [6,7]. The buffer used for these experiments was 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % polyethylene glycol (PEG) 8000 in PEG 20,000-coated acrylic cuvettes. S2238 was used as substrate and the residual thrombin activity was quantified by measuring the initial rate of hydrolysis from the linear increase in absorbance at 405 nm as a function of time under conditions wherein less than 10% substrate is consumed. Briefly, a solution of 10 µL CDSO3 at concentrations ranging from 0.1–8600 µM was diluted with 963 µL of buffer and 7 µL of 0.23–1.07 µM thrombin and mixed well. This was followed by addition of 20 µL S2238 (2 mM) and the initial rate was rapidly measured. Logistic equation 1 was used to fit the dose dependence of residual thrombin activity to obtain IC_{50} of inhibition.

$$Y = Y_o + \frac{Y_M - Y_o}{1 + 10^{(\log[LMWL]_0 - \log IC_{50})}} \quad (\text{Eq. 1})$$

In this equation, Y_M and Y_o are the maximum and minimum values of the fractional residual thrombin activity; IC_{50} is the concentration of the inhibitor that results in 50% inhibition of enzyme activity. Sigmaplot 8.0 (SPSS, Inc. Chicago, IL) was used to perform nonlinear curve fitting in which Y_M , Y_o , and IC_{50} were allowed to float.

2.3 Analysis of Serine Protease Sequences and X-ray Crystal Structures

The primary amino acid sequence of thrombin, factor Xa, factor IXa and factor VIIa was retrieved from the ExPASy web server (<http://www.expasy.org>). A multiple alignment was performed on the sequences using ClustalX version 2.0.11 with default parameters [14]. A representative crystal structure for each protease was retrieved from the RCSB Protein Data Bank (PDB; <http://www.pdb.org>). SYBYL 8.1 (Tripos, Inc., St. Louis, MO) was used to prepare the structures and figures derived from the parent PDB file. All redundant subunits, water, cofactors, inhibitors and other ligands were removed from the crystal structures prior to molecular surface calculations. In addition, the SYBYL Mutate Monomers function was used to assign coordinates to the incompletely resolved arginine and lysine side chain atoms. Hydrogen atoms were added using the Add Hydrogens feature. The serine proteases were then aligned spatially (Fit Monomers function) using the backbone atoms of the amino acid positions corresponding to the basic arginine and lysine residues of thrombin exosite 2, i.e., Arg⁹³, Arg¹⁰¹, Arg¹⁶⁵, Arg²³³, Lys²³⁶ and Lys²⁴⁰. Unless otherwise noted, the chymotrypsin numbering system [15] is used to facilitate identification of corresponding residues in the chymotrypsin-like serine protease family members.

3. Results and Discussion

3.1 Specific Residues of Thrombin are Involved in Recognition of CDSO3

Our earlier work led to the conclusion that CDSO3 does not bind in the active site or exosite 1 of thrombin [7]. Experiments using exosite 2 ligands such as unfractionated heparin and LMWH showed good competition with CDSO3 [7]. Likewise, sulfated LMWLs were also found to compete with heparin for binding to human plasmin also [8], which supported exosite 2-like site as the prime site of CDSO3 recognition. Yet, exosite 2 of thrombin is a rather large ellipsoidal domain spanning an area of approximately 20×30 Å². It consists of

several basic residues, e.g., Arg⁹³, Arg¹⁰¹, Arg¹⁶⁵, Arg²³³, Lys²³⁵, Lys²³⁶, and Lys²⁴⁰, some of which are known to play important roles in heparin binding. Interestingly, Arg⁹³ and Arg¹⁰¹ of this group are adjacent to a hydrophobic patch, which we reasoned may be important for recognizing the aromatic rings of CDSO3. To identify the key residues of exosite 2 that are important for CDSO3 binding, we studied inhibition of a library of thrombin mutants. The library included single-site replacement of basic residues of exosite 2 (identified above) with alanine. In addition, additional residues near exosite 2 including Arg⁹⁷, Lys¹⁶⁹, Arg¹⁷³, and Arg¹⁷⁵ were also studied. The preparation and characterization of these thrombin mutants have been described earlier [12,13]. Direct inhibition of these mutants by CDSO3 was studied in a manner similar to that used for wild-type thrombin [7].

The dose-response profiles of eleven mutants studied here were essentially identical to the recombinant wild-type enzyme (Fig. 2). The measured IC_{50} s for Arg⁹³Ala and Arg¹⁷⁵Ala thrombin mutants were 313 and 275 nM, which were 7–8-fold greater than that of recombinant wild-type thrombin (39 nM) (Table 1). In contrast, alanine replacement at Arg⁹⁷, Arg¹⁰¹, Lys²³⁵, Lys²³⁶, or Lys²⁴⁰ positions did not affect the CDSO3 inhibition potency (0.9–1.5-fold effect), while substitutions at the 165, 169, 173 and 233 positions introduced a 2.2–3.7-fold decrease in inhibition potency (Fig. 3A).

The maximal inhibition efficacy of CDSO3 across these thrombin mutants ranged from 47–82% in comparison to 58% observed for the recombinant wild-type form. These efficacies are generally similar to that noted earlier for human plasma thrombin and human plasma plasmin (~80%) [7,8].

To assess the effect of multiple replacements on CDSO3 inhibition potency, double and triple replacement at the 93, 97, and 101 positions were studied. Replacing arginines at both 93 and 97 positions with alanines shifted the dose-response profile significantly to the right in comparison to both the wild-type enzyme as well as either single-site mutant (Table 1, Fig. 3B). The measured IC_{50} for the 93,97-double mutant was 679 nM, which indicated an increase of ~17-fold and ~2-fold over wild-type and Arg⁹³Ala enzymes, respectively. Introducing alanines for arginines at 93, 97, and 101 positions led to an increase in IC_{50} of ~36-fold from the wild-type form (Fig. 3B). The efficacy of inhibition for the double and triple mutants was comparable to other thrombins studied in this work (~80%, Table 1).

3.2 Three-dimensional structures reiterate Arg⁹³ and Arg¹⁷⁵ as key points for interaction with CDSO3

Our earlier work shows that CDSO3 potently inhibits thrombin and factor Xa, moderately inhibits plasmin, and does not inhibit factor VIIa and factor IXa [7]. Each of these enzymes is a trypsin-related serine protease with considerable three-dimensional structural similarity and we reasoned that CDSO3 could be reliably expected to bind in domains similar to exosite 2 of thrombin.

To evaluate the specificity of recognition of enzymes, the primary sequences of human thrombin, factor Xa, factor IXa, factor VIIa, and plasmin were aligned using ClustalX (see Figure S1 in Supplementary Material for full alignment). The enzymes display considerable similarity in length and sequence, as expected, especially in the exosite 2 region (Table 2). The alignment indicates that thrombin and factor Xa are the most closely related enzymes in terms of their exosite 2 regions with percent identity as high as 36%. Both enzymes display electropositive residues at 93, 165, 169, 236 and 240 positions (Fig. 4). Although factor Xa contains a neutral residue at 175, the side chain of Lys169 can effectively mimic the positive charge of Arg¹⁷⁵ of thrombin (not shown). Likewise, human plasmin contains Lys⁶⁴⁵ (plasminogen numbering) that is equivalent to Arg⁹³ of thrombin in three-dimension (not shown), while also containing basic residues at 101, 175 and 233 positions. In contrast,

neither factor IXa nor factor VIIa possess an arginine at 93 or at 175, and display much poorer sequence similarity to thrombin ($\leq 18\%$, Table 2).

Measurement of partition coefficient ($\log P$), a simultaneous measure of solubility in aqueous and hydrophobic media, has suggested that sulfated LMWLs are considerably hydrophobic [9] in addition to being anionic. Analysis of hydrophobicity maps of the five enzymes being studied here shows that Arg⁹³ of thrombin and factor Xa is located on the periphery of a fairly extensive hydrophobic patch made by side chains of Leu⁶⁰, Pro^{60B}, Ile⁸⁸, Ile⁹⁰, Tyr⁹⁴ and Trp⁹⁶ (Fig. 4). These residues can potentially interact with the aromatic rings of CDSO3. Such an extensive hydrophobic patch is almost non-existent in factors VIIa and IXa (not shown). Thus, it appears that CDSO3 recognizes a specific sub-site within exosite 2 that affords interaction with hydrophobic side-chains near appropriate basic residues (Fig. 4), which explains the observed selectivity of recognition by sulfated LMWLs.

3.3 CDSO3-based allosteric inhibition of thrombin offers a powerful avenue to discover new anticoagulants

Discovering allosteric modulators of protein function is challenging and yet highly sought after considering that this form of regulation is suggested to offer finer control necessary for therapeutic success [2,16]. Despite this expectation, allosteric modulators of cardiovascular enzymes, other than heparin and heparinoids, have not been vigorously pursued [17,18]. In this regard, sulfated LMWLs offer excellent promise. The identification of the site of binding of these novel molecules on thrombin implies by analogy a corresponding site on factor Xa and plasmin, which offer new avenues for discovering anti- as well as pro-coagulants [11].

The availability of the library of recombinant thrombin variants offered a unique tool in identifying the sub-domain consisting of Arg⁹³, Arg¹⁷⁵, and Arg¹⁶⁵ as important for binding. The results also show that Lys²³⁵, Lys²³⁶ and Lys²⁴⁰ are not important for CDSO3 recognition. Heparin, on the other hand, recognizes Lys²³⁶, Lys²⁴⁰, Arg⁹³, Arg¹⁰¹ and Arg²³³ [19] in that order of importance. Interestingly, the magnitude of binding defect introduced by these single mutations is significantly different for the two polyanions. Whereas Arg²³⁶ and Arg²³³ replacement introduced ~43- and 8-fold defects for heparin binding [20], the maximal defect for CDSO3 binding was ~8-fold for Arg⁹³Ala thrombin. These results reveal interesting similarity as well as differences between the two polyanionic molecules and highlight the importance of aromatic backbone of CDSO3. More importantly, identification of this sub-domain implies that for the first time detailed molecular modeling studies can be initiated to design advanced molecules based on CDSO3 structure.

This work also explains the selectivity of enzyme recognition by CDSO3 and other sulfated LMWLs. Although each enzyme studied to date is a trypsin-based serine protease, the ability to selectively target thrombin and factor Xa is intriguing. Analysis of the three-dimensional structure of thrombin indicates that Arg⁹³, the key residue for interaction with CDSO3, forms a 'ridges' adjacent to a well-defined hydrophobic 'valley' (Fig. 4). Such a well-defined hydrophobic region is present only in thrombin, factor Xa, and plasmin, and not in factors IXa and VIIa. We hypothesize that sulfated LMWLs, which contain multiple aromatic rings, utilize this hydrophobic region in addition to positive charge of Arg⁹³ and other basic residues for interacting with select group of coagulation enzymes.

The reason why Lys²³⁵, Lys²³⁶ and Lys²⁴⁰ do not play a more important role for CDSO3 as compared to that for LMWHs is not clear. The hydrophobic 'valley' similar to that noted adjacent to Arg⁹³ (described above) is not present near these contiguous residues, which is in line with our hypothesis that an optimal combination of charge and hydrophobic character, and not just polyanionic character, in CDSO3 induces recognition of coagulation

enzymes. We believe that this hypothesis could be advantageously exploited for other coagulation enzymes so as to design highly specific CDSO3-based structures that target only one enzyme in an allosteric manner.

The observation that double and triple mutations progressive weaken the inhibition potential of CDSO3 is interesting considering that individual single mutations (Arg¹⁰¹Ala or Arg⁹⁷Ala) do not appear affect potency to a significant degree. In either case, Arg⁹³, the key residue for CDSO3 recognition, was intact, which possibly suppresses the loss.

Finally, sulfated LMWLs may offer a unique advantage with regard to the mechanism of action. The exosite 2-mediated allosteric regulation of thrombin offers a sound possibility of an antidote strategy with non-inhibitory sulfated carbohydrates. For example, small oligosaccharides that bind in exosite 2 without inducing much direct inhibition, such as sucrose octasulfate [21], may serve as effective antidotes of CDSO3-based anticoagulants. This would impart considerable safety to anticoagulant therapy based on sulfated LMWLs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Sulfated LMWLs are novel allosteric inhibitors of human thrombin
- Site-directed thrombin mutants were used to identify the site of binding of sulfated LMWLs.
- Arg⁹³ and Arg¹⁷⁵ were found to be key residues for recognizing sulfated LMWLs
- The identification of this novel allosteric site on thrombin will greatly assist the design of new anticoagulants.

CDSO3

$(R_3 = \text{OH or } \text{OSO}_3^-;$
 $X = \text{H or OH or } \text{OSO}_3^-;$
 $Y = \text{H or } \text{COO}^-)$

Figure 1.

Structure of CDSO3, a sulfated LMWL. CDSO3 primarily contain β -O-4, β -5, β - β and 5-5 inter-residue linkages (shown shaded). X and Y are substituents at the α - and β - positions and may be -H, -OH, or $-\text{OSO}_3\text{Na}$ and -H or COONa , respectively. R may be either -OH or $-\text{OSO}_3\text{Na}$. Variations in these substituents, inter-residue linkages and configuration at α - or β - positions generate a large number of sequences.

Figure 2.

Direct inhibition of recombinant thrombins by CDSO3 in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1 % polyethylene glycol (PEG) 8000. Shown is the inhibition of wild-type thrombin (○); Arg⁹³Ala thrombin (△), and Arg¹⁷⁵Ala thrombin (□). Solid lines represent dose-response fits of Eq. 1 to the data to derive Y_M , Y_0 and IC_{50} . See text for details.

Figure 3.

Effect of single-site (A) and multi-site (B) replacement of electropositive residues of exosite 2 of thrombin on the direct inhibition potency of CDSO3. The ratio of IC_{50} values of the mutant to that of recombinant wild-type is shown. 'Dbl mut' refers to Arg⁹³Ala + Arg¹⁰¹Ala thrombin. Likewise, 'Tpl mut' refers to Arg⁹³Ala + Arg⁹⁷Ala + Arg¹⁰¹Ala thrombin. Error bars shown are ± 1 S.E.

Figure 4.

The overall structure of thrombin (PDB ID = 1TB6), highlighting the electropositive amino acid residues studied in this work. The carbon atoms of these residues are color-coded based on the magnitude of the decrease in inhibition by CDSO3 upon mutation to alanine (red: > 5-fold, green: 2–5-fold, white: < 2-fold), indicating the putative binding site of CDSO3. This region encompasses part of anion-binding exosite 2 and features two distinct hydrophobic patches (transparent yellow and magenta surfaces). Amino acids contributing to the hydrophobic surfaces are rendered in stick representation and include Leu⁶⁰, Pro^{60B}, Ile⁸⁸, Ile⁹⁰, Tyr⁹⁴ and Trp⁹⁶ (yellow surface) and Tyr⁸⁹, His⁹¹ and Pro⁹² (magenta surface). The protein backbone is shown as a ribbon and the 60-loop (blue), 99-loop (yellow), 170-loop (cyan) and C-terminal region (magenta) are highlighted.

Table 1
Inhibition of Recombinant Wild-Type and Mutant Thrombins by CDSO3.^a

	<i>IC</i> ₅₀ (nM)	<i>Y</i> ₀	<i>Y</i> _M	ΔY
WT	39 ± 3	47 ± 1	105 ± 2	58 ± 2
R93A	313 ± 20	21 ± 2	103 ± 2	82 ± 9
R97A	37 ± 4	49 ± 2	102 ± 3	53 ± 4
R101A	37 ± 5	33 ± 3	101 ± 4	68 ± 9
R165A	144 ± 27	27 ± 5	95 ± 4	68 ± 15
K169A	85 ± 12	33 ± 3	98 ± 3	65 ± 8
R173A	115 ± 15	52 ± 2	99 ± 2	47 ± 3
R175A	275 ± 19	34 ± 2	100 ± 2	66 ± 5
R233A	86 ± 15	30 ± 5	99 ± 4	69 ± 14
K235A	44 ± 6	32 ± 3	101 ± 4	69 ± 9
K236A	58 ± 3	20 ± 1	102 ± 1	82 ± 5
K240A	57 ± 6	27 ± 2	103 ± 4	76 ± 9
R93,97A	679 ± 108	25 ± 5	96 ± 5	71 ± 18
R93,97,101A	1416 ± 121	18 ± 3	98 ± 3	80 ± 16

^aInhibition studies were performed in 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 2.5 mM CaCl₂ and 0.1% PEG 8000 in PEG 20000-coated acrylic cuvettes. S2238 was used as substrate and the residual enzyme activity in the presence of CDSO3 was assessed by measuring the initial rate of substrate hydrolysis at 405 nm. Logistic equation 1 was used to fit the dose dependence of the residual enzyme activity to obtain log *IC*₅₀, *Y*_M and *Y*₀ values. See Methods for details.

^bCorresponds to the difference between *Y*_M and *Y*₀ values

^cRepresents ±1 S. E.

Table 2
Comparison of Electropositive Residues of Exosite 2-like Domains in Selected Human Coagulation Enzymes.^a

	93	97	101	165	169	173	175	233	235	236	240	% ^b
Thrombin	R	R	R	R	K	R	R	R	K	K	K	100
Factor Xa	R	E	F	R	K	S	I	A	L	K	R	36
Plasmin	R/K^c	— ^d	K	N	N	N	R	R	V	T	G	27
Factor IXa	N	A	H	R	L	K	T	R	V	N	E	18
Factor VIIa	T ^d	G ^d	H	T	L	S	N	Q	I	E	K	9

^aMultiple sequence alignment of coagulation serine proteases was performed using ClustalX version 2.0.11 with default parameter settings. See text for details. The ExPASy protein codes were P00734 (human thrombin), P00742 (human factor Xa), P00747 (human plasmin), P00740 (human factor IXa), and P08709 (human factor VIIa). Residues shown in bold indicate identity with thrombin.

^bPercent identity with thrombin, based on the amino acid positions listed in this table.

^cThese consecutive residues (R644 and K645; plasminogen numbering) are found in approximately the same location as thrombin R93 and thus can likely substitute for it.

^dDue to the widely varying loop lengths in the 99-loop region, the amino acid residue assignment is somewhat arbitrary.