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ORIGINAL ARTICLE

Correlation of factor IXa subsite modulations with effects on substrate discrimination

P. F. NEUENSCHWANDER, K. J. DEADMOND, K. ZEPEDA and J. RUTLAND
Center for Biomedical Research, The University of Texas Health Science Center at Tyler, Tyler, TX, USA

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Summary. *Background:* A key feature of factor IXa is its allosteric transformation from an enzymatically latent form into a potent procoagulant. Although several small molecules have been found to be capable of partially affecting FIXa function (i.e. ethylene glycol, Ca^{2+}, and low molecular weight heparin [LMWH]), the resulting modest changes in peptidolytic activity have made the study of their mechanisms of action challenging. As these effects provide hints about potential regulatory forces that may be operational in the full expression of FIXa coagulant activity, their description remains of great interest. Studies of crystal structures have yielded insights into the structural changes induced by these effectors, but there remains a paucity of information to correlate any given structural change with specific consequences for FIXa function. *Objectives:* To correlate structural changes induced by these modulators with defined consequences for FIXa substrate discrimination and function. *Methods:* A peptidomics-based mass spectrometry (MS) approach was used to examine the patterns of hydrolysis of four combinatorial chemistry-derived pentapeptide libraries by FIXa under various conditions in a soluble, active enzyme system. *Results:* Ethylene glycol specifically altered the S3 subsite of FIXa to render it more tolerant to side chains at the P3 substrate position, whereas Ca^{2+} enhanced tolerance at the S2 subsite. In contrast, LMWH altered both the S2 and S1′ subsites. *Conclusions:* These results demonstrate the role of plasticity in regulating FIXa function with respect to discrimination of extended substrate sequences, as well as providing crucial insights into active site modulations that may be capitalized on by various physiologic cofactors of FIXa and in future drug design.

Keywords: enzymology, factor IXa, hemophilia, structure, substrate, thrombosis.

Correspondence: Pierre F. Neuenschwander, Center for Biomedical Research, The University of Texas Health Science Center at Tyler, 11937 US Highway 271, Tyler, TX 75708, USA.
Tel.: +1 903 877 7678; fax: +1 903 877 7675.
E-mail: pierre.neuenschwander@uthct.edu

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Introduction

Blood coagulation factor IXa is a member of the serine protease family of enzymes and a crucial part of the intrinsic pathway of blood coagulation [1,2]. Its deficiency, or that of its major protein cofactor, FVIII, results in hemophilia B or A, respectively. Conversely, excessive FIXa activity has been implicated in thrombotic disease [3]. The maximal procoagulant activity of FIXa is observed only when it is in a quaternary complex with three essential cofactors: activated FVIII (FVIIIa), Ca^{2+}, and an anionic phospholipid membrane surface. The resulting lipid-bound enzyme–cofactor complex (commonly referred to as the intrinsic 'tenase' complex) exhibits an overall 10^9-fold enhancement in activity towards its native substrate, FX. The numerous structural mechanisms and molecular details that define this dramatic enhancement in procoagulant activity are not well defined, and remain the subject of much investigation.

An interesting feature of FIXa that separates it from other blood coagulation proteases is its comparatively low peptidolytic activity even in the context of the fully assembled tenase complex, which exhibits full procoagulant (proteolytic) activity. The combination of these seemingly contradictory enzymologic characteristics strongly supports the notion of a poorly formed (but functional) FIXa catalytic center (as revealed in available crystal structures [4,5]) whose deficiencies are corrected for or overcome by cofactor-induced molecular effects outside of the catalytic center. In this view, these cofactor-induced changes result in the elaboration of sufficient proteolytic activity towards the macromolecular substrate FX to support coagulation, with little (although in some cases measurable) elaboration of activity towards small substrates (such as peptides). Although intriguing, these characteristics have severely hampered studies of FIXa enzymatic activity and its mechanisms with traditional enzymologic techniques. Whereas numerous studies have been published concerning the esterolytic and amidolytic activities of thrombin, FXa, and FVIIa, there is a comparative paucity of such studies with FIXa. Instead, these characteristics have led to the majority of enzymologic studies of FIXa focusing on the elucidation of the macromolecular effects of cofactor binding. This has produced a complex (although still incomplete) model of enzymatic enhancement that involves a fascinating array of interconnected macromolecular mechanisms (reviewed in [6,7]).

Ethylene glycol has previously been shown to enhance the activity of FIXa and some other clotting serine proteases, although not all of them [8–10], through an unknown mechanism. Although the action of ethylene glycol as such does not have physiologic significance for FIXa clotting activity, its ability to affect FIXa activity is intrinsically of great interest, owing to the difficulties that have been encountered in gaining insights into the function and regulation of this cryptic serine protease. Thus, understanding the molecular details of this effect, as well as those of other modulators, and correlating them with precise functional consequences is an essential prerequisite for their future exploitation in drug design. This information will help to provide a deeper understanding and appreciation of the role of enzyme plasticity and subtle structural modulations in defining proteolytic activity and regulation.

Given the subtle nature of the proposed modulations of the dramatic increase in FIXa enzyme activity [11–13], it remains critical to accurately compare and correlate observed structural perturbations with actual effects on enzyme function in the context of the native and physiologically relevant enzyme. This remains a daunting task. The goal of the present study was to gain further insights into such correlations by examining specific functional consequences of perturbations that occur in the FIXa active site upon stimulation with various known, and relatively simple, effectors: Ca^{2+} [14], low molecular weight heparin (LMWH) [13,15], and ethylene glycol [9,10,13]. The approach involved the merging of combinatorial chemistry and peptidomics to probe the FIXa active site for alterations induced by these various modulators in an active, soluble enzyme system with native FIXa. Although the results generally confirm the alteration of specific enzyme subsites, as observed in static crystal structures, there are some critical differences that may have implications for future drug design and our understanding of FIXa modulatory effects and conformational activation events.

Materials and methods

Reagents

Ammonium bicarbonate, calcium chloride and ethylene glycol were from Fisher Scientific (St Louis, MO, USA). The LMWH Lovenox was from Sanofi-Aventis (Bridgewater, NJ, USA). Trypsin was from Sigma-Aldrich (St Louis, MO, USA). Bovine serum albumin (BSA; fraction V – fatty acid-free) was from CalBiochem (La Jolla, CA, USA). Trifluoroacetic acid (TFA) and 2,5-dihydroxybenzoic acid (DHB) were from Fluka Analytical (Sigma-Aldrich Chemie, Buchs, Switzerland). Native human FIXaβ (FIXa) was from Haematologic Technologies (Essex Junction, VT, USA). Spectrozyme FIXa, Spectrozyme t-PA and Spectrozyme FXIIa substrates were from American Diagnostica (Greenwich, CT, USA). CBS 31.39 and CBS 48.03 substrates were from Diagnostica Stago (Parsippany, NJ, USA).

Peptide libraries

The four pentapeptide libraries used were derived from the extended reactive sequence of human antithrombin, AGRSL [13]. This peptide is referred to as the base peptide (AT3.2). Each library contained a pool of 20 peptides containing one degenerate position where each of the 20 amino acids had been incorporated (Table 1) by the use of combinatorial chemistry and L-isomers throughout. The Arg at the P1 position was left unchanged to allow hydrolysis by trypsin-like serine proteases. To better mimic a continuous peptide chain and reduce potential artificial effects of charged termini, all peptides were synthesized with N-terminal acetyl (Ac) and C-terminal amide (Am) blocking groups (Ac-AGRSL-Am).

All peptides and peptide libraries were synthesized by New England Peptides (Gardner, MA, USA), using Fmoc chemistry, and were HPLC-purified. Their spectral masses were determined to be within 0.1% of the exact expected molecular masses as provided. Typical reactions used 1 mM of the entire peptide library, or $\sim$ 50 μM of each peptide sequence.

Peptide library hydrolysis reactions

Unless otherwise indicated, enzyme reactions were performed with either 10 nM trypsin or 25 nM FIXa in 100 mM NH_4HCO_3 (pH 8) at 37 °C for either 1 h (trypsin) or 18 h (FIXa) with the indicated additives. Reactions were terminated upon drying to completion in a Speed-Vac (Thermo Savant, Asheville, NC, USA), and then frozen at − 20 °C until mass spectrometry (MS) analysis.

Chromogenic enzyme assays

Assays were performed in 96-well microplates (untreated polystyrene; Corning Inc., Corning, NY, USA) at ambient temperature, essentially as previously described [13,15]. Standard final reaction conditions were 25 nM FIXa in 50 mM Tris-HCl (pH 8.5), 0.1 M NaCl, 0.1% (w/v) BSA, and 5 mM $CaCl_2$, unless otherwise indicated.

Table 1 Peptide libraries based on AT3.2 pentapeptide

	Amino acid identity and position*				
	P3	P2	P1↓	P1′	P2′
AT3.2 base peptide:	A	G	R	S	L
P3 library	X	G	R	S	L
P2 library	A	X	R	S	L
P1′ library	A	G	R	X	L
P2′ library	A	G	R	S	X

*Each library was synthesized by the use of combinatorial chemistry with L-isomers of amino acids and blocked with an acetyl moiety on the N-terminus and an amide moiety on the C-terminus. Each peptide library contains all 20 possible amino acids substituted at the position denoted by an X. The P1 Arg was not varied, to allow for peptide hydrolysis (arrow) by FIXa (a trypsin-like serine protease).

MS

MS analyses of peptide libraries [16] were performed with a Kratos Axima-CFR Kompact MALDI-TOF MS instrument (Shimadzu Corporation, Kyoto, Japan). Calibration of the instrument was performed with a 1 : 1 mixture of undigested and trypsin-digested AT3.2 peptide (1 mM). Immediately prior to MS analysis, reaction samples were reconstituted in 10 μL of 0.1% TFA. One microliter of each sample was then mixed directly on a stainless steel sample plate with an equal volume of DHB matrix [17] (10 mg mL^{-1} in 60% acetonitrile/0.1% TFA) and allowed to crystallize at room temperature. MS spectra were recorded in the positive reflectron mode with the pulsed extraction mass set to the center of the range of masses being investigated. Under these conditions, hydrolysis products were identifiable at levels as low as 5 ng. To facilitate proper identification of products, a spotting level of 25 ng was used throughout.

Data analysis

Kratos Analytical (Kratos Analytical Inc., Chestnut Ridge, NY, USA) software (KOMPACT, v 2.4.1) was used to visualize MALDI-TOF MS spectra and identify substrate and product peaks, as well as to perform spectral smoothing by averaging and to calculate peak areas. Results were internally normalized for each identified peptide sequence by taking into consideration the remaining unhydrolysed (intact) peptide mass peak and the mass peak of the unique hydrolysis product resulting from normal hydrolysis C-terminal to the P1 Arg. This is defined by Equation 1:

$$\%\text{Hydrolysis} = \left(\frac{\text{Hydrolysed}}{\text{Unhydrolysed} + \text{Hydrolysed}}\right) \times 100 \qquad (1)$$

Each library and condition was tested in triplicate, and hydrolysis percentages are reported as averages. This internal normalization procedure takes into account and corrects for all variations in individual peptide levels, reaction mixtures, and sampling/pipetting, as well as laser-shot variations. In order to compare values across peptide libraries, values of % hydrolysis were secondarily normalized to the observed hydrolytic activity towards the base peptide sequence AGRSL, which was present in each library (internal control). This step takes into account and corrects for any variation in FIXa activity between experiments, and is reported as fractional reactivity (Equation 2).

$$\text{Fractional Reactivity} = \frac{\%\text{Hydrolysis}}{\%\text{Hydrolysis}_{\text{AGRSL}}} \qquad (2)$$

Results

Previous studies by us showed that an Ac-AGRSL-Am pentapeptide substrate (AT3.2) could sense changes in FIXa enzymatic activity as induced by Ca^{2+}, heparin, and ethylene glycol [13]. To further elucidate the basis for the effects of these modulators on the active site of FIXa, we used the AT3.2 peptide as a base peptide sequence to generate four individual peptide libraries (Table 1) by combinatorial chemistry (New England Peptides). Each library represented a pool of 20 different pentapeptide sequences with a single degenerate amino acid in the sequence at either the P3, P2, P1′ or P2′ position. The P1 position was left unchanged (Arg) in all libraries to allow for hydrolysis by serine-like proteases. As the base peptide sequence AGRSL was present in all four libraries, 77 different peptide sequences were theoretically represented (see below).

Characterization of the peptide sequences represented in each library

A sample of each library was mock-digested (no enzyme), dried, dissolved in 0.1% TFA, and spotted on a MALDI target at 500 ng per spot (roughly 25 ng of each peptide). A typical MS spectrum (P3 library) is shown in Fig. 1A. Although substantial variations were observed in peak height and area for the different peptides present in each library, the differences were reproducible and consistent between experiments. Thus, it is likely that this reflects differences in peptide synthesis levels and laser desorption/ionization efficiency as opposed to

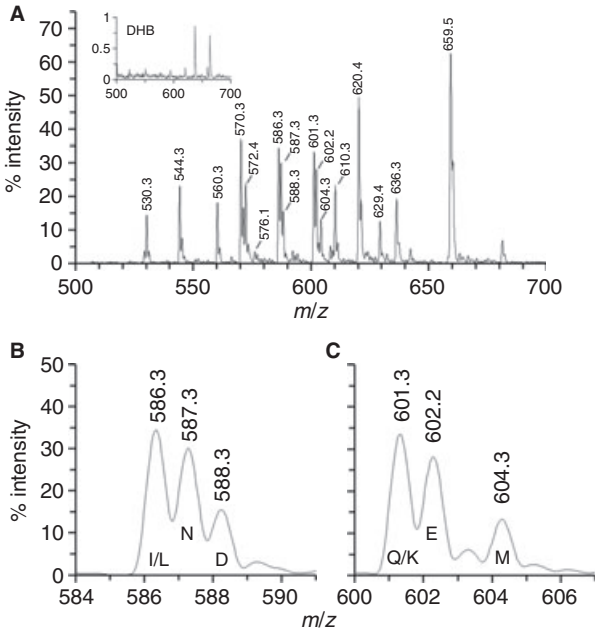

Fig. 1. MALDI-TOF MS analysis of the intact AT3.2-P3 peptide library. A typical MS spectrum of the AT3.2-P3 library is shown for illustrative purposes (A). Values of % intensity were normalized to that of the 2,5-dihydroxybenzoic acid (DHB) matrix peak (m/z of 154, not shown). The MS spectrum in the absence of peptide (essentially the DHB matrix alone) revealed insignificant masses in the range examined (inset). Each peptide library revealed 17 peptide m/z peaks corresponding to 19 of the 20 possible peptide sequences (as labeled; see Tables S1–S4). Owing to Ile and Leu having identical masses, as do Gln and Lys, peptides containing these residues at the degenerate position have masses indistinguishable from each other at the resolution of this system (B, C).

experimental variability. Of all the expected peptides in each library, only the Thr version of each degenerate peptide was not identified to a significant extent (although Ac-AGRST-Am was identified at an extremely low level in the P2′ library). In addition, as Ile and Leu have identical masses, and Gln and Lys have masses that are indistinguishable from each other at the resolution of this system, both of these peptide pairs yielded single peaks (Fig. 1B,C). None of these peptide peaks was observed when the peptide was left out of the reaction mixture, and minimal background signal was observed from the matrix/buffer system (Fig. 1A, inset).

Hydrolysis of the libraries by FIXa

The peptide hydrolysis MS spectrum of each of the libraries was determined for FIXa alone, and in the presence of 5 mM Ca^{2+}, 10 μM LMWH, 30% ethylene glycol, or combinations. MS analyses of each reaction produced well-resolved groups of substrate and product peaks (Figs 2 and S1). As expected, the numbers and levels of products observed were different under each condition. Generally, the Arg version of each degenerate peptide was present only at low levels, and produced sporadic results. Likewise, Cys-containing peptides, although observable, were present only at low levels, and the libraries contained extremely variable levels of oxidized dimers that were not well hydrolysed. Thus, results from sequences containing Thr, Cys or Arg in the degenerate position were omitted from subsequent analysis. In addition, as the *m*/*z* of peptides containing either Ile/Leu or Gln/Lys in the degenerate position were indistinguishable from each other (cf. Fig. 1 and Tables S1–S4), the areas for these peaks were considered to be representative of the peptide pairs as opposed to the individual sequences. Thus, the final analysis focused on 15 *m*/*z* peaks (17 peptide sequences) for each library, or 65 total different peptide sequences out of the 77 theoretically possible. The representation of peptide sequences in each library was close to expected levels, but did exhibit some variability (Fig. S2), necessitating normalization procedures (Equations 1 and 2).

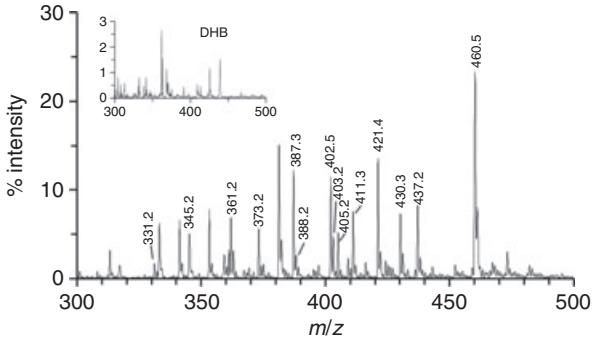

Fig. 2. MALDI-TOF MS analysis of the hydrolysed AT3.2-P3 peptide library. A typical MS spectrum of the AT3.2-P3 library after hydrolysis with FIXa in the presence of 30% ethylene glycol is shown for illustrative purposes. Values of % intensity were normalized to that of the 2,5-dihydroxybenzoic acid (DHB) matrix peak (*m*/*z* of 154, not shown). The MS spectrum for the reaction mixture in the absence of peptide (essentially the DHB matrix alone) revealed insignificant masses in the *m*/*z* range examined (inset). Each peptide library and condition examined revealed different product *m*/*z* peaks, but all products were readily identifiable on the basis of their expected *m*/*z* values (see Tables S1–S4). The analysis focused on 15 potential *m*/*z* product peaks representing 17 peptide sequences.

Correlations between FIXa subsite modulations and substrate discrimination

As expected because of its poor activity, FIXa alone showed extremely low and sporadic hydrolysis of all four libraries (not shown). However, the addition of any of the three effectors substantially increased and stabilized hydrolytic activity, allowing consistent MS measurements to be made. For each library, an average value for fractional reactivity (reactivity normalized to AGRSL) was determined under each condition to allow interlibrary comparisons to be made. The analysed data are shown in Fig. 3A as the overall fractional reactivity observed with each library under each condition examined. The results revealed complex responses to multiple effectors, but fairly clear responses to singular effectors. The predominant

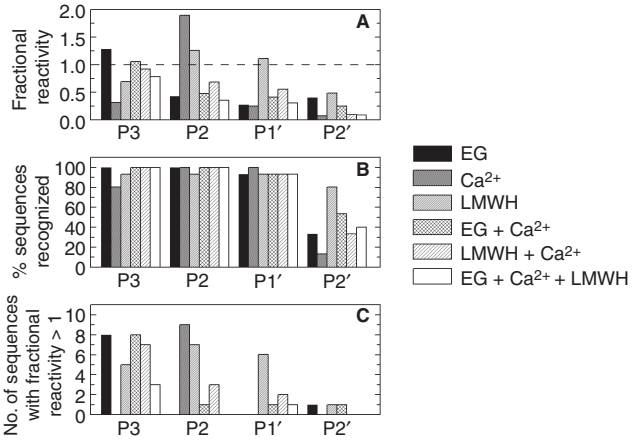

Fig. 3. Modulation of substrate discrimination by FIXa. (A) The average fractional reactivity of each of the four different peptide libraries (P3, P2, P1′, and P2′) for FIXa in the presence of various known FIXa effectors is shown. The effectors are: 30% v/v ethylene glycol (EG), 5 mM Ca^{2+} (Ca^{2+}), and 10 μM low molecular weight heparin (LMWH, Lovenox). Values for fractional reactivity were obtained from mean values of % hydrolysis ($n = 3$), which were then normalized to the mean % hydrolysis of the base AGRSL peptide present in each library (arbitrarily defined as 100% hydrolysis and fractional reactivity of 1.0, dotted line) (see Equations 1 and 2). (B) The percentage of sequences recognized under each condition examined is indicated for each library. The values are based on the 17 peptide sequences that were included in the analysis of each library; sequences containing Thr, Cys or Arg at the degenerate position were omitted (see Results). Most conditions examined produced at least detectable levels for the majority of sequences. The P2′ library showed the fewest products, probably because of solubility issues with this library. (C) To gain further insights into overall enhancements in reactivity over the AGRSL base peptide sequence, the number of sequences (out of the 15 total product peaks examined) with a fractional reactivity greater than 1.0 are shown. These results indicate that, for the main modulations observed, the average values of fractional reactivity in (A) are not simply attributable to very large increases in reactivity towards a few sequences, but rather are indicative of overall increases in reactivity towards the majority of sequences in the libraries.

effect of ethylene glycol alone was observed with the P3 library, and the predominant effect of Ca^{2+} alone was observed with the P2 library. LMWH was found to have the most global effect, but this was predominantly observed with the P2 and P1′ libraries. Despite these differences, the total number of peptides in each library that were hydrolysed to any extent remained fairly constant, with most conditions yielding identifiable products for 80–100% of the different peptide sequences represented in the libraries (Fig. 3B). The obvious exception to this was the P2′ library, for which the number of products observed was significantly lower. The pattern observed in Fig. 3A was reproduced when plots of the number of peptides in each library whose fractional reactivity was > 1.0 were examined for each condition (Fig. 3C). This analytic view supports the notion that for those conditions where average fractional reactivity increased, this was attributable to an overall increase in the tolerance of variation and not just an increase in the fractional reactivity of a few preferred sequences. Together, these data reaffirm the targeting of the beneficial effects of ethylene glycol, Ca^{2+} and LMWH to specific FIXa subsites as sensed by the various peptide degenerate amino acid positions. In addition, this analysis demonstrates the effects of these modulations on enhancing global substrate side chain accommodation (substrate discrimination) of the FIXa subsites rather than in defining single-substrate specificity or recognition.

Correlations between FIXa subsite modulations and rates of substrate hydrolysis

The above results demonstrate a specific functional consequence of each FIXa effector that is focused on a specific and different FIXa subsite. Although these modulations clearly affect substrate discrimination, they may or may not also be involved in affecting subsequent enzymatic events in peptide hydrolysis. As the observed effects of Ca^{2+} and ethylene glycol were focused on specific subsites that are N-terminal to the scissile bond (non-prime side), we were able to examine the potential kinetic consequences of these defined modulations by using commercially available chromogenic substrates. The use of these substrates, as opposed to the pentapeptide substrates used above, greatly facilitates subsequent kinetic analyses as well as providing valuable validation of the above observations by an alternative method. The chromogenic substrates used are given in Table 2, and were obtained from several suppliers. All are *p*-nitroanilide-based substrates with small, defined differences in the P4, P3 and P2 positions, thus allowing direct comparisons to be made concerning the effects of the various modulators.

Heparin alone had little to no effect in altering the initial rates of hydrolysis of these substrates (not shown). In contrast, ethylene glycol enhanced the activity of FIXa towards all of the substrates in a substrate sequence-dependent manner. For the four substrates with Gly at the P2 position, maximal FIXa activity was observed in the presence of 30–40% (v/v) ethylene glycol (Fig. 4), consistent with previous observations [8,10]. The sole substrate containing a bulky phenylated Gly in the P2 position (Spectrozyme FIXa) exhibited substantially different ethylene glycol dependence, with peak activity being observed at significantly lower levels of ethylene glycol (20–25% v/v). Of additional interest is the fact that only the two substrates containing CH_3SO_2 in the P4 position (as the blocking group) exhibited the maximal 17-fold enhancement in reactivity. The remaining substrates, with a different or no blocking group, exhibited only a two-fold to four-fold enhancement in reactivity. Considering that each of the chromogenic substrates use the D-isomer of Leu in the P3 position, the subsequent opposite steric positioning of the N-terminal blocking group in the active site makes this result fully consistent with effects of ethylene glycol on the S3 subsite as described above (see Discussion). Examination of the kinetic parameters of substrate hydrolysis indicated that this effect was probably attributable to a change in K_m for the substrate, with no measurable change in k_{cat} (Fig. S3).

An examination of the Ca^{2+} dependence of substrate hydrolysis (Fig. 5) revealed that whereas Ca^{2+} effectively enhanced the ability of FIXa to hydrolyse all of the substrates to some degree, hydrolysis of Spectrozyme FIXa was affected differently from that of the other substrates (Fig. 5B). This was

Table 2 Sequences of chromogenic substrates examined with FIXa*

	Sequence				
Substrate	P4	P3	P2	P1↓	P1′
Spectrozyme FIXa		D-L	phG	R	*p*-Nitroanilide
CBS 31.39	CH_3SO_2	D-L	G	R	*p*-Nitroanilide
CBS 48.03	CH_3OCO	D-L	G	R	*p*-Nitroanilide
Spectrozyme t-PA	CH_3SO_2	D-chT	G	R	*p*-Nitroanilide
Spectrozyme FXIIa		D-chT	G	R	*p*-Nitroanilide

*Each tripeptidyl chromogenic substrate sequence is presented from the N-terminus to the C-terminus. The CH_3SO_2 and CH_3OCO moieties on three of the substrates are N-terminal blocking groups. All of the chromogenic substrates contained a D-isomeric amino acid at the P3 position, and three of the five substrates incorporated modified amino acids: phenylated Gly (phG) or cyclohexyl-Thr (chT). Hydrolysis by trypsin-like serine proteases occurs at the amide bond between the P1 Arg and the P1′ *p*-nitroanilide moiety (arrow).

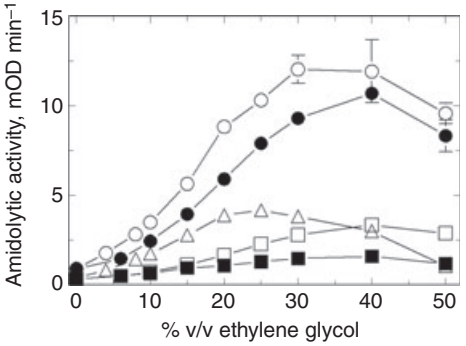

Fig. 4. Substrate sequence specificity of the ethylene glycol effect on FIXa chromogenic activity. Initial rates of hydrolysis of CBS 31.39 (○), Spectrozyme t-PA (●), CBS 48.03 (□), Spectrozyme FXIIa (■) and Spectrozyme FIXa (△) were measured under standard conditions with increasing concentrations of ethylene glycol (% v/v). The sequence of each substrate is given in Table 2. Points are means of triplicate determinations ± standard error, where 1 mOD $= 1 \times 10^{-3}$ absorbance units at 405 nm.

most apparent in the absence of ethylene glycol, where FIXa activity was greater towards Spectrozyme FIXa than to all of the other substrates. This result is consistent with Ca^{2+} binding enhancing the side chain tolerance of the S2 subsite in FIXa as observed above (cf. Fig. 3).

Discussion

This study addresses a crucial gap in the study and understanding of FIXa activity and regulation. By examining the changes in hydrolysis patterns of four pentapeptide libraries by FIXa in the presence of three different known small molecule effectors of FIXa (ethylene glycol, Ca^{2+}, and LMWH), we have been able to begin establishing correlations between the structural alterations induced by these relatively simple effectors [12,15] and specific functional consequences with respect to substrate discrimination by FIXa.

Interpretation of the results and comparison across libraries required a secondary normalization procedure of the data to determine the fractional reactivity as compared with the base peptide sequence AGRSL (Equation 2). Thus, potential effects on the reactivity of the AGRSL peptide with FIXa under the various conditions are not observable. These normalization steps were required because of the variability observed in absolute % hydrolysis values between MALDI-TOF MS experiments (although hydrolysis patterns remained constant) and the variation in the level of each peptide in the libraries.

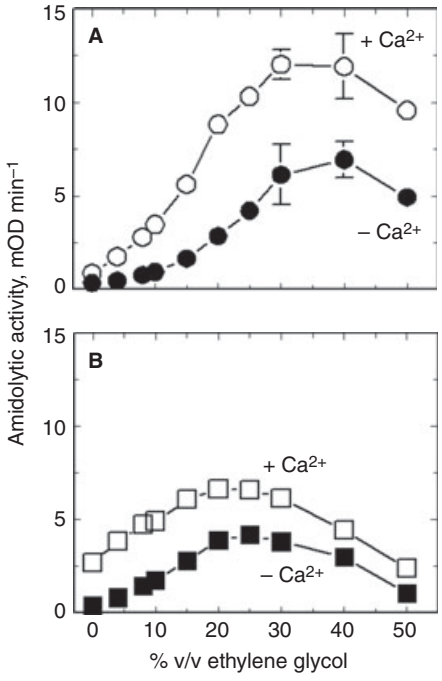

Fig. 5. Substrate sequence specificity of the Ca^{2+} effect on FIXa chromogenic activity. Initial rates of hydrolysis of CBS 31.39 (A) or Spectrozyme FIXa (B) were measured under standard reaction conditions and the indicated levels of ethylene glycol in the presence of either 5 mM Ca^{2+} (○, □) or 2.5 mM EDTA (●, ■). Points are means of triplicate determinations ± standard error, where 1 mOD = 1×10^{-3} absorbance units at 405 nm.

Although this precludes the drawing of conclusions with respect to substrate sequence preferences within libraries or effects on initial rates of hydrolysis, this procedure does allow the examination of changes in overall patterns of reactivity across libraries, which is the main premise of the study.

Modulation of the FIXa S3 region

Our results demonstrate that the ethylene glycol-induced alterations in FIXa predicted by fluorescence studies [8] and observed in structural studies [12] can be correlated with enhanced tolerance of variation of L-amino acid side chains at the P3 position of peptide substrates. This result provides direct evidence of a specific functional consequence of S3 modulation by ethylene glycol, and supports a model of this effector producing a more side chain-accessible S3 region. We have previously reported that ethylene glycol induces specific changes in the fluorescence of Fl-D-FPR-labeled FIXa that are not observed with other similarly labeled blood clotting serine proteases [8]. This suggested that the effect of ethylene glycol on FIXa was attributable, at least in part, to specific structural alterations of FIXa near the dye. Although the fluorescein dye of the inhibitor and the CH_3SO_2 moiety of the chromogenic substrates used here are located at the P4 position of the linear peptide sequence, on the basis of the presence of a D-isomeric amino acid at the P3 position in both of these peptides, as well as on the intrinsic rotatability of the amide bond, it is quite feasible (and in fact highly probable) that the moiety at the P4 position of these non-natural peptides senses changes in the region of FIXa that is typically defined as S3. This is supported by molecular docking simulations (Fig. 6), and is consistent with the proposed binding site of ethylene glycol being adjacent to the S3 region in crystal structures of FIXa [12]. The docking configuration of the free AGRSL peptide is supported by the recent study from the Huntington laboratory describing the crystal structure of AT-bound FIXa in the presence of Ca^{2+} and pentasaccharide [18].

Modulation of the FIXa S2 region

Our data clearly demonstrate that Ca^{2+} modulates FIXa discrimination of the P2 substrate residue. This is probably attributable to effects on the S2 subsite that result in an increased tolerance of variation of side chain structures at the P2 position. The S2 region is more highly defined than the S3 and S4 regions of FIXa, owing to the more rigorous requirements of positioning of the P2 substrate residue for proper scissile bond reactivity. Available FIXa crystal structures suggest that this region is largely defined by the 99-loop (orange region in Fig. 6A). Thus, our results would seem to implicate modulations of the 99-loop in the response to ionic Ca^{2+} binding. This is somewhat surprising, given that only minimal structural changes were observed in the 99-loop upon Ca^{2+} removal from crystals [12]. However, the FIXa used in the crystal studies was a truncated and mutated form of *Escherichia coli*-expressed FIXa (FIXa_3) in which part of the

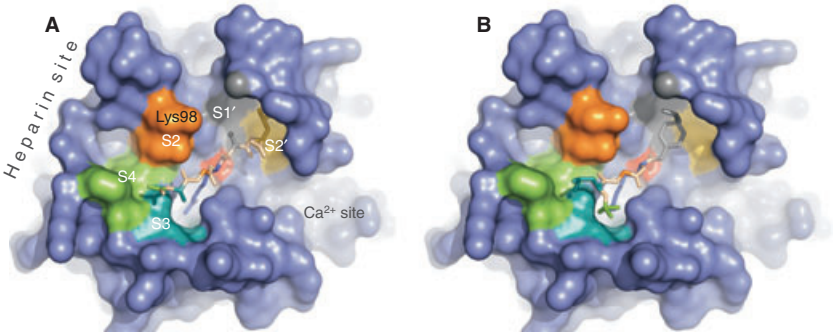

Fig. 6. Molecular docking models of FIXa and peptide substrates. The AGRSL base peptide (A) or CBS 31.39 (B) was docked into the active site of FIXa (Protein Data Bank code 1RFN) with AUTODOCK VINA [19], and displayed by use of the PYMOL Molecular Graphics System, Version 1.4, Schrödinger, LLC. Putative subsites in FIXa are indicated (S4, S3, S2, S1′, and S2′), and are color-coded to match the corresponding amino acid position in the peptide. The P1 Arg is shown in blue inserted into the primary S1 specificity pocket of FIXa. The Ca^{2+}-binding site and the LMWH (heparin)-binding regions in FIXa are indicated. Lys98, which is part of the 99-loop and forms part of the S2 region, has been shown to be directly modulated by heparin binding [15]. The orientations of the P3 and P4 residues are reversed in CBS 31.39 (which incorporates a D-isomeric Leu in the P3 position) as compared with the AGRSL peptide. This is predicted to orient the N-terminal CH_3SO_4 moiety of CBS 31.39 (green stick structure in [B]) towards the FIXa S3 region. The active site Ser195 is indicated by the red surface in the center of the active site. Both dockings resulted in the reactive carbonyl of the substrate being placed adjacent to the reactive hydroxyl of Ser195 (~ 3 Å), consistent with what would be expected for a productive enzyme–substrate complex.

99-loop had been altered (specifically, Lys98 was mutated to Thr). Additionally, the effects of Ca^{2+} may have been somewhat masked in those studies, as a result of Ca^{2+} extraction from crystals that had been preformed using covalently inhibited FIXa, locking the enzyme into a specific conformation. The potential consequences of these numerous significant differences from the native soluble and physiologic form of FIXa are difficult to judge, and may very well result in conformational perturbations that are substantially different from those that occur in the native molecule during substrate hydrolysis. Our combined use of wild-type FIXa, peptide substrates composed of natural amino acids and an assay system based on substrate hydrolysis (rather than enzyme inhibition) can be considered to more accurately reflect the molecular changes that occur in the S2 region in response to Ca^{2+}, and that are required for FIXa enzymatic function.

Modulation of the FIXa active site 'upper rim'

Our previous studies have shown that LMWH binding to FIXa allosterically modulates Lys98 in the 99-loop in such a way as to remove its hindrance of the active site, thus allowing reactivity of FIXa with the Kunitz-type inhibitor BPTI. The results obtained with small synthetic chromogenic substrates did not recapitulate this effect, indicating that, perhaps, the movement of Lys98 may be too far removed from the immediate active site to directly affect their reactivity. However, studies with the AGRSL libraries show that LMWH binding does indeed alter the substrate discrimination of FIXa, by increasing the side chain tolerance of variation at the P2 and P1′ positions. This apparent discrepancy can be explained by the lack of a proper P1′ side chain and the presence of small side chains at the P2 position in the chromogenic substrates, which would preclude sensing changes in both the S2 and S1′ regions. Overall, the results are consistent with LMWH altering the configuration of the 'upper rim' of the FIXa active site. This could be entirely attributable to movement of the Lys98 side chain, as previously described [15]. Interestingly, the combination of Ca^{2+} and heparin negated their individual effects, suggesting that competing modulations probably occur. Indeed, although LMWH was found not to enhance the hydrolysis of chromogenic substrates, we have observed a significant and consistent reduction of the rate of hydrolysis of 1 mM Spectrozyme FIXa (phenylated Gly at P2) when 10 μM LMWH (Lovenox) is added to a mixture of 25 nM FIXa and 5 mM Ca^{2+} in the absence of ethylene glycol (not shown). This is probably not merely a result of Ca^{2+} depletion by its binding to heparin, as Ca^{2+} is present in large molar excess (roughly 500-fold) over LMWH (33-fold molar excess over saccharide units; Lovenox is 15 saccharide units in length).

The disparities between substrate recognition, substrate discrepancy and substrate reactivity are well recognized in enzymology, but the relationships between them remain ambiguous. Thus, it is important to note that the results of this study do not necessarily suggest greater reactivity of FIXa towards specific substrate sequences, and conclusions regarding such substrate preferences are not warranted on the basis of the data presented. The results do, however, demonstrate the role of plasticity in regulating FIXa function. These mechanisms may be used in part and/or in various combinations by different physiologic cofactors, modulators and substrates to induce the desired final proteolytic functionality of FIXa. The ability to artificially and differentially modulate FIXa functionality also highlights the great potential for the use of these various molecular effects in the rational design of drugs to control and modulate FIXa reactivity for treatment of either thrombosis or hemophilia.

Although hydrolysis of peptides is typically monitored with HPLC, previous studies have demonstrated the use of MS as a higher-throughput and rapid method that is suitable for the analysis and identification of peptide fragments in mixtures of peptides [16]. The present study demonstrates that this type of

analysis is extendable to larger libraries as well as being amenable to MALDI-TOF MS instrumentation. Thus, the technique is suitable for identification of known peptide masses within complex reaction mixtures, and the peaks are well removed from contaminant peaks and the DHB matrix peaks. This study provides a basis for future comparison and a novel methodology for approaching FIXa enzymology. Future studies of this type examining the modulatory effects on FIXa by using hydrolysable peptidyl substrate libraries may help to unravel the complex and subtle mechanisms involved in regulating the activity of this cryptic serine protease.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Typical full MALDI-TOF MS spectrum of peptide library substrate and products.
Figure S2. Fractional representation of peptide sequences in libraries.
Figure S3. Kinetic analysis of the effect of ethylene glycol on FIXa reactivity.
Figure S4. Fractional reactivities of sequences in response to ethylene glycol.
Figure S5. Fractional reactivities of sequences in response to Ca^{2+}.
Figure S6. Fractional reactivities of sequences in response to LMWH.
Table S1. Predicted monoisotopic masses of peptides and hydrolysis products for the AT3.2-P3 library (Ac-XGRSL-Am).
Table S2. Predicted monoisotopic masses of peptides and hydrolysis products for the AT3.2-P2 library (Ac-AXRSL-Am).
Table S3. Predicted monoisotopic masses of peptides and hydrolysis products for the AT3.2-P1′ library (Ac-AGRXL-Am).
Table S4. Predicted monoisotopic masses of peptides and hydrolysis products for the AT3.2-P2′ library (Ac-AGRSX-Am).

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