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Macromolecular substrate affinity for free factor VIIa is independent of a buried protease domain N-terminus ☆

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Abstract

The initial recognition and binding of macromolecular substrates by factor VIIa (FVIIa) in complex with tissue factor has been shown to be mediated by areas distinct from the active site (so-called exosites). The present aim was to shed light on whether the N-terminal tail of the protease domain of FVIIa influences factor X (FX) binding, and whether the zymogen-like conformation of free FVIIa has a decreased affinity for FX compared to the active conformation. Two derivatives of FVIIa, one (FFR-FVIIa) with a stably buried N-terminus representing the active conformation of FVIIa and one (V154G-FVIIa) with a fully exposed N-terminus representing the zymogen-like conformation, were used as inhibitors of FVIIa-catalyzed FX activation. Their inhibitory capacities were very similar, with K_i values not significantly different from the K_m for FX. This indicates that the conformational state of the N-terminus does not affect FX binding or, alternatively, that the activation domain including the N-terminal insertion site is easily shifted to the stable conformation ensuing FX docking to the zymogen-like conformation. The net outcome is that FX binding to the zymogen-like form of FVIIa does not appear to be impaired.

Keywords: Factor VIIa; Activation pocket; N-terminus; N-terminal insertion; Zymogen-like; Substrate affinity

Coagulation factor VII(a) (FVII(a)) patrols the vasculature for exposed tissue factor (TF). Free FVIIa has very low biological activity due to an incomplete zymogen-to-enzyme conversion after internal cleavage, and the appearance of TF upon vascular injury mediates the localization of FVIIa and allosteric stimulation of its activity [1]. This extreme cofactor dependence of FVIIa ensures that the blood clotting cascade is triggered at the appropriate place and time [2]. Ensuing complex formation between the two proteins, FVIIa exerts its physiological hemostatic function by generating factors IXa (FIXa) and Xa (FXa) from the corresponding zymogens, reactions that initiate blood clotting [3]. Pharmacologically, administration of recombinant FVIIa (NovoSeven) results in supraphysiological levels of the enzyme, and buoyed by the high number of molecules FVIIa can produce considerable amounts of FXa in a TF-independent manner. This intrinsic ability, and the widespread clinical use of NovoSeven, makes a characterization of the properties of free FVIIa relevant [4].

Crystal structures reveal that FVIIa and TF interact in the binary complex using a large interface [5,6]. One point of contact, between Met306 in FVIIa and TF, appears to be particularly important for the allosteric effect on FVIIa and its active site [7,8]. TF binding also leads to stabilization of the activation pocket and thereby facilitates the insertion of the N-terminal tail of the protease domain of FVIIa and the salt bridge formation with the side chain of Asp343 [9]. This salt bridge is crucial for the TF-induced FVIIa activity enhancement.

The interactions of FIX and FX with FVIIa · TF have only been mapped to some extent by site-directed mutagenesis. A common substrate-interactive region on TF has been identified [10,11], which also is involved in the

☆ *Abbreviations:* FIX(a), (activated) factor IX; FVII(a), (activated) factor VII; FX(a), (activated) factor X; TF, tissue factor.

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interaction with FXa [12,13]. All studies pinpoint the previously identified lysine residues 165 and 166, and some of the surrounding residues as being critically important for cofactor activity [14,15]. The Gla domain of FVIIa [16,17] and the N-terminal Gla and epidermal growth factor-like domains of the protein substrates [18–21] have also been implicated in interactions within the ternary complex. Less is known regarding interactions within the FVIIa · TF · FX/FIX complex more distant from the membrane surface. For instance, so far no substrate-interactive residues have been identified in the N-terminal domain of TF [22]. However, alanine scanning has pinpointed several residues in FVIIa which influence FX activation by FVIIa · TF [23], but how this is accomplished has in most cases not been investigated. Certain FVIIa protease domain residues, such as Arg290 [24] and Glu296 [25], may be selectively important for macromolecular substrate processing through an involvement in binding and/or turnover rate. Interestingly, these residues are included in the most potent inhibitory FVIIa-derived peptide found in a study of FVIIa · TF-catalyzed FX activation [26]. With time, it has become increasingly evident that substrate (FX) binding to the FVIIa · TF complex is dictated by exosites distant from the active site cleft of FVIIa. More recently, this has been confirmed by the failure of mutations of the P1 residue in FX and in subsites in the catalytic cleft of FVIIa to reduce substrate affinity, as well as by the demonstration that active site and exosite occupation results in non-competitive and competitive inhibition of FX activation, respectively [27,28].

To date, studies of the interactions between FX and FVIIa have been carried out in the presence of TF and are consequently influenced by the allostery by which TF runs FVIIa. This context is physiologically most relevant, but during rFVIIa therapy free FVIIa is a significant player and this merits investigations of its substrate interactions. Free FVIIa is in equilibrium between a predominant, zymogen-like conformation and a catalytically competent state. One difference, presumably of several, between the two conformations is the configuration of the amino-terminus, being buried and engaged in a salt bridge with Asp343 in the active conformation and solvent-accessible in the zymogen-like conformation. To shed light on whether the binding of FX is affected by the configuration of the N-terminus, we have used derivatives of FVIIa forced into one or the other form as inhibitors of FVIIa-catalyzed FX activation. Our data also clarify whether FVIIa in the zymogen-like conformation is incapable of activating its macromolecular substrates due to abolished enzymatic activity, compromised binding, or both.

Materials and methods

Proteins and reagents. FVIIa [29] and D-Phe-L-Phe-L-Arg (FFR)-FVIIa [30] were obtained as previously described. The V154G mutation was introduced into FVII using the QuikChange kit (Stratagene, La Jolla, CA, USA), the forward primer 5′-CCC CAA GGC CGA ATT GGG GGC AAG GTG TGC CCC-3′ (base substitution in italic and the affected codon underlined) and the complementary reverse primer. The wild-type FVII expression plasmid pLN174 was used as the template [31]. Methods for vector preparation, cell transfection, and protein production and purification have been described [32]. V154G-FVII was activated by incubation with FIXaβ (10%, w/w) overnight at 37 °C, followed by removal of FIXaβ by chromatography on an F1A2 (anti-FVIIa) immunoaffinity column. FX, FXa, and FIXaβ were purchased from Enzyme Research Laboratories (South Bend, IN, USA) and the peptidyl substrate S-2765 was from Chromogenix (Mölndal, Sweden). Stock solutions of the proteins to be used in the FX activation assays were buffer-exchanged into 50 mM Hepes, pH 7.4, containing 0.1 M NaCl and 5 mM $CaCl_2$ using a NAP-10 column, and further dilutions were made in the same buffer containing 0.1% (w/v) bovine serum albumin. After buffer exchange, the concentrations of FVIIa, FFR-FVIIa, V154G-FVIIa, and FX were determined by absorbance measurements at 280 nm using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) using extinction coefficients of 1.32 (FVIIa) and 1.16 (FX) for a 1 mg/ml protein solution.

FX activation assays. For the determination of the K_m for FX, 100 nM FVIIa was incubated with 1–15 μM FX in 100 μl for 20 min at room temperature. The FX activation was stopped by the addition of 50 μl of 50 mM Hepes, pH 7.4, containing 0.1 M NaCl and 20 mM EDTA. The FXa generated was measured after the addition of 50 μl of a 2 mM solution of S-2765 as the rate of hydrolysis of chromogenic substrate during a 5 min period and converted to [FXa] using a FXa standard curve from 0.5 to 5 nM. Absorbance measurements were performed at 405 nm using a SpectraMax 190 kinetic microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Data were corrected for the intrinsic activities of FVIIa and FX. In the competition experiments, 400 nM FVIIa in 25 μl was mixed with 2–32 μM FFR-FVIIa or 2–16 μM V154G-FVIIa in 50 μl, followed by the addition of 25 μl of a 600 nM FX solution. This gives final concentrations of 100 nM FVIIa, various competitor concentrations, and 150 nM FX. The mixtures were incubated for 120 min at room temperature, and termination of FX activation and quantification of the formed FXa were carried out as described above. Data were corrected for the intrinsic amidolytic activities of the individual proteins (FVIIa, FX, and competitor incubated with FX). Separate experiments showed that the generation of FXa at 150 nM FX was linear over time in the interval 20–240 min. The amount of FXa generated was also virtually linearly dependent on the FX concentration in the range 30–150 nM.

Results and discussion

Traditionally, estimates of the binding energy between substrate and enzyme are based on K_m values. With free FVIIa, the K_m for FX represents the affinity of FX for catalytically competent FVIIa, that is for the FVIIa molecules that release FXa rather than return FX to the substrate pool, because the K_m value is derived from the dependence of the amount of FXa generated on the FX concentration. Thus, the interaction between zymogen-like or non-productive FVIIa and FX escapes quantification in such an activity-based assay. TF facilitates the insertion of the N-terminus of the protease domain of FVIIa [9], an insertion that is pivotal for enzymatic activity in the trypsin family of serine proteases [33], and concomitantly stimulates the amidolytic activity of FVIIa more than 20-fold. Evidently TF traps or stabilizes the active FVIIa conformation shifting the equilibrium towards this state. The vast majority (>95%) of the free FVIIa molecules thus appear to be in a zymogen-like conformation, which includes an exposed N-terminus of the protease domain, and unable to activate

FX. In order to estimate the affinity of FX for both active and zymogen-like FVIIa, of which FVIIa is a mixture, two derivatives of FVIIa were employed. First, incorporation of an irreversible inhibitor into the active site of FVIIa to generate FFR-FVIIa stabilizes the insertion of the N-terminus [30]. Only modification with this type of inhibitor yields a population of inactivated FVIIa molecules with a stably inserted N-terminus suitable as a representative of the catalytically competent, active conformation [34]. The use of this type of derivative is also justified by the previous finding that FX docking with the active site cleft of FVIIa does not contribute to substrate affinity [27]. Second, mutation of residue 154 to Gly reduces the hydrophobic interactions with the activation pocket and generates a very flexible N-terminal tail which is virtually impossible for FVIIa to bury [35], even when bound to TF, making this variant a good representative of the zymogen-like conformation. Even though FFR- and V154G-FVIIa mimic the active and inactive FVIIa conformation, respectively, at least with respect to the different states of the N-terminus of the protease domain, both preparations have very low enzymatic activity. They can therefore be used to compete with FVIIa for FX resulting in inhibition of FVIIa-catalyzed FX activation. The residual FVIIa activity of FFR-FVIIa, due to incomplete inhibition, was about 0.4%, and in our hands V154G-FVIIa activated FX approximately 40-fold slower than did FVIIa.

First, we determined the K_m value for FX to be approximately 23 μM (Fig. 1). The accompanying k_{cat} value was $1.9 \times 10^{-4}\ s^{-1}$, yielding a catalytic efficiency of about $8\ M^{-1}\ s^{-1}$. The inhibitory capacities of FFR-FVIIa and V154G-FVIIa were subsequently assessed at a FX concentration (150 nM) far below K_m. Because the FXa generation was linearly dependent on the FX concentration in the 30–150 nM range, a reduction in FX activation by, for instance, 30% in the presence of competitor could be directly translated into a 30% reduction in available FX. The two competitors displayed similar IC_{50} values ($\sim K_i$ under our experimental conditions), about 19 μM for FFR-FVIIa and 14 μM for V154G-FVIIa, not far from the K_m value of FVIIa for FX (Fig. 2). These data suggest that a solvent-accessible, as in V154G-FVIIa, and a stably buried, as in FFR-FVIIa, N-terminus of the protease results in indistinguishable affinities for FX. It can also be inferred that zymogen-like FVIIa fails to activate FX exclusively due to catalytic inability and not due to impaired substrate binding.

Models of the ternary TF · FVIIa · FXa complex suggest that the activation domain of FVIIa, including the N-terminal insertion site, is at the interface between FVIIa and FX(a) [36,37]. Even though this does not necessarily imply a measurable contribution to the binding energy, combining the present findings with these models, and assuming that free and TF-bound FVIIa interact identically with FX, suggests that a rapid transition of FVIIa to the active conformation may occur upon or after the initial docking of FX. It should be kept in mind that V154G-FVIIa is a very poor FX activator, and if such a transition occurs it would mean that the V154G mutation has a detrimental effect on the catalytic efficiency of FVIIa even after N-terminal insertion. However, the similar affinity of FX for both conformations of FVIIa would allow for FX binding to the prevailing, zymogen-like form followed by a conformational change in FVIIa that permits FX activation. Why, then, is free FVIIa a poor activator of FX? It is conceivable that, in the absence of TF, the N-terminus is seldom correctly inserted into the protease domain. Alternatively, the N-terminus escapes and free FVIIa spontaneously falls back into the zymogen-like conformation before it has cleaved FX. These possibilities are supported by successful stabilization of the inserted N-terminus by mutagenesis of FVIIa and its functional manifestation as an increased intrinsic activity [32,38]. The increased rate by which some of these superactive FVIIa variants, in the free form, activate FX can presumably to a large extent be explained by

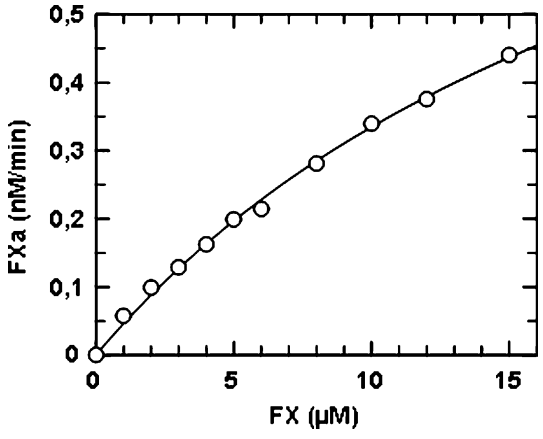

Fig. 1. FVIIa-catalyzed FX activation. The rate of FXa formation by 100 nM FVIIa was measured in incubations with 1–15 μM FX. A K_m value of 23 μM was derived from these data. See Materials and methods for experimental details.

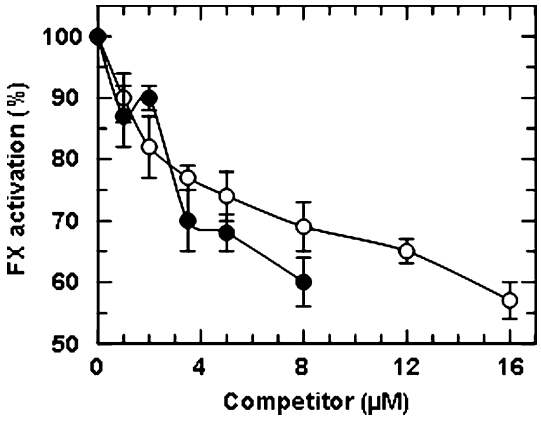

Fig. 2. Effects of FFR-FVIIa (open symbols) and V154G-FVIIa (closed symbols) on FVIIa-catalyzed FX activation. From these data (mean ± SEM, $n = 2$), IC_{50} values of approximately 19 and 14 μM, respectively, were derived for the competitors. See Materials and methods for experimental details.

a shift in the equilibrium between the two conformational states of FVIIa towards the active one. This is most evident in FVIIa variants which display a much larger increase in proteolytic than in amidolytic k_{cat} relative to wild-type FVIIa, a property indeed accompanied by a stably buried N-terminus. Altogether, this supports the well-established notion that the conformational status of the N-terminus affects the processing of macromolecular substrates by FVIIa, but our data with FFR-FVIIa and V154G-FVIIa demonstrate that it has little, if any, influence on the affinity for these protein substrates.

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