Cell. Mol. Life Sci. 65 (2008) 953 – 963
1420-682X/08/060953-11
DOI 10.1007/s00018-007-7480-5

Cellular and Molecular Life Sciences

Review

Cofactor-induced and mutational activity enhancement of coagulation factor VIIa

O. H. Olsen* **and E. Persson**

Haemostasis Biochemistry, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv (Denmark),
Fax: +45 44434547, e-mail: oho@novonordisk.com

Received 18 October 2007; received after revision 12 November 2007; accepted 14 November 2007
Online First 8 December 2007

Abstract. Coagulation factor VIIa (FVIIa) is an atypical member of the trypsin family of serine proteases. It fails to attain spontaneously its catalytically competent conformation and requires its protein cofactor tissue factor (TF) to accomplish this. Over a number of years, this unique behaviour of FVIIa has prompted investigations of the TF-induced activation mechanism and the zymogenicity determinants in factor VIIa. Factor VIIa has gained additional interest in the past decade because of its development into a clinically useful haemostatic agent. Here, we present an overview of the current knowledge about the TF-induced allosteric activation of FVIIa and the various molecular approaches to enhance the intrinsic activity and efficacy of FVIIa.

Keywords. Factor VIIa, tissue factor, factor VIIa analogue, factor X activation, allostery, intrinsic activity, zymogen-like.

Introduction

Upon vascular injury, the cell surface receptor tissue factor (TF) is exposed to the circulating blood and binds coagulation factor VIIa (FVIIa) and any extravascular, preformed FVIIa:TF complexes are also exposed to the blood [1–4]. This complex is the trigger for blood coagulation through factor IX (FIX) and factor X (FX) activation and FVII autoactivation on the TF-bearing cells. The FX activation to FXa in turn generates enough thrombin to activate platelets and convert factors VIII (FVIII), V and XI to their activated forms [5, 6]. The thrombin-activated platelets provide a perfect template for binding of FVIIIa and FIXa (tenase complex), leading to further activation of FX, and binding of factor Va and FXa (prothrombinase complex) resulting in large-scale thrombin generation and a fibrin clot. In addition to initiating blood coagulation, the FVIIa:TF complex also induces signalling events influencing cellular responses and pathophysiological processes [3, 7].

FVIIa consists of a trypsin-like serine protease domain and an N-terminal, disulphide-linked light chain composed of a membrane-binding γ-carboxyglutamate-rich domain (Gla domain) and two epidermal growth factor (EGF)-like domains (EGF1 and EGF2 (Fig. 1) [8, 9]. Apart from binding to TF, the light chain domains help to position the protease domain and its active site at a proper distance above the membrane surface [10]. Like other serine proteases in the trypsin family, FVII is secreted as a zymogen (single chain) and needs endoproteolytic cleavage of the peptide bond preceding position 153

* Corresponding author.

{16}[1] (trypsinogen numbering in brackets) to become catalytically active. In trypsin, the newly formed N terminus spontaneously inserts into a cavity, termed the activation pocket, resulting in the formation of a salt bridge with Asp^{194} (residue 343 in FVIIa) [11]. The formation of the salt bridge leads to maturation of the catalytic apparatus, i.e. the substrate-binding cleft and the oxyanion hole. However, FVIIa has a very low catalytic activity due to poor N-terminal insertion. TF allosterically facilitates this process [12] and markedly accelerates the activity of FVIIa towards its physiological substrates [13].

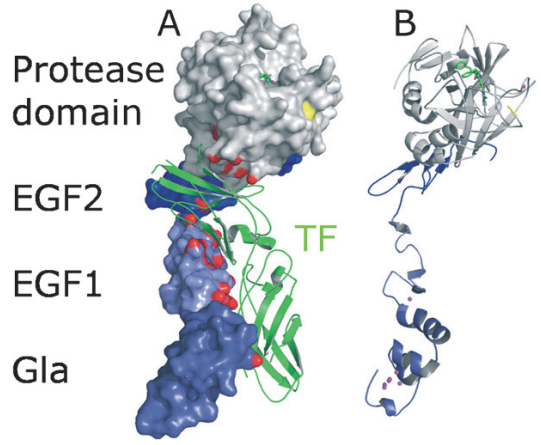

Figure 1. Overview of the FVIIa:TF complex (Protein Database entry code: 1dan.pdb). (*A*) The structure of FVIIa is shown in a surface-rendered representation while that of TF is shown as a cartoon model in green. The domain structure of FVIIa is depicted using different colors: the Gla domain in deep blue, the EGF1 domain in light blue, the EGF2 domain in blue and the protease domain in grey. A covalent inhibitor is present in the active site (green carbon atoms). The atoms in FVIIa closer than 3.5 Å from TF are in red. (*B*) Cartoon model of FVIIa. Ca^{2+} ions are shown as magenta spheres: seven are bound to the Gla domain, and one in each of the EGF1 and the protease domains.The structures were drawn using PyMol v0.99.

Patients with haemophilia A and haemophilia B lack or have dysfunctional FVIII and FIX, respectively. These patients are usually treated by replacement therapy using factors of either plasma or recombinant origin. A considerable percentage of these patients, however, develop inhibitory antibodies against the administered protein which makes replacement therapy inefficient [14]. Recombinant FVIIa (rFVIIa, NovoSeven®, Novo Nordisk A/S, Bagsværd, Denmark) constitutes an efficient substitution therapy for the treatment of this group of patients. At pharmacological concentrations, rFVIIa is able to bind to thrombin-activated platelets and is capable of activating factor X directly, in the absence of TF, thereby generating thrombin and bypassing FVIII and FIX. Outside the haemophilia indication, NovoSeven is approved for use in patients with congenital FVII deficiency and with a platelet disorder, Glanzmann's thrombasthenia, and it is continuously being evaluated in a variety of other medical and drug-induced conditions [15–17]. The natural next step in drug development would be to search for rFVIIa analogues with higher intrinsic activity and an improved clinical efficacy. Such analogues may prove to be clinically more useful because they would induce haemostasis more rapidly and potentially result in the formation of a more rigid clot. This review describes the structure and natural activation mechanism of FVIIa, the pharmacological mechanism of action of rFVIIa, the approaches used to date to generate various classes of improved rFVIIa analogues and the properties of these new molecules.

Structural properties

The X-ray crystallographic structure of FVIIa in complex with TF [18] was a major breakthrough in the understanding of the molecular details of this pivotal interaction and the first structure solution of an enzyme-cofactor complex in the coagulation cascade. Earlier, alanine scanning mutagenesis had revealed sites in FVIIa important for interaction with TF [19]. The structure of FVIIa is depicted in Figure 1 with the trypsin-like protease domain shown in grey and the N-terminal light chain in blue. The interaction between FVIIa and TF is characterized by a large interface divided into several domains. The structural characteristics of each domain of FVIIa are described in some detail below.

The Gla domain including the neighbouring hydrophobic stack consists of the first 45 amino acid residues [20]. The structure of the FVIIa Gla domain is similar to that of other vitamin K-dependent coagulation factors including prothrombin, FIX, FX and protein C. The posttranslationally γ-carboxylated first nine glutamic acid residues in FVIIa (referred to as Gla residues [21]) are clearly resolved in the structure [18], while Glu^{35}, the tenth glutamic acid residue and the last potential carboxylation site, is disordered and possibly not (completely) modified. The Gla residues are responsible for Ca^{2+} binding, which induces a significant structural reorganization into a membrane-binding conformer [22–24]. The Gla domain binds seven calcium ions, six of which are arranged in a

[1] Trypsinogen numbering is as follows: Arg^{152}, 15; Ile^{153}, 16; Val^{158}, 21; His^{193}, 57; Asp^{242}, 102; Glu^{296}, 154; Met^{298}, 156; Leu^{305}, 163; Met^{306}, 164; Cys^{310}, 168; Gln^{313}, 170A; Ser^{314}, 170B; Asn^{322}, 175; Cys^{329}, 182; Lys^{337}, 188; Asp^{338}, 189; Asp^{343}, 194; Ser^{344}, 195; His^{373}, 224; Phe^{374}, 225.

planar fashion. When bound to a lipid membrane, the array of calcium ions is presumably placed in the plane of the phospholipid headgroups. Binding of calcium to Gla^{6} and Gla^{7} induces an ω-like conformation of the first 10 residues which exposes Phe^{4}, Leu^{5} and Leu^{8} to the solvent in a manner suitable for binding to cell membranes containing phophatidylserine [22–26]. Despite the homology and a high degree of sequence identity between the Gla domains of the coagulation factors, the binding affinity for synthetic anionic phospholipid membranes varies by several orders of magnitude [27]. Hence, the dissociation constant for bovine prothrombin was determined to be 150-fold lower than that of bovine FVIIa despite a sequence identity of 70%. Biochemical data and X-ray structure elucidation suggest specific interactions with phosphatidylserine [22]. The FVIIa interaction with TF in this region is mainly hydrophobic and involves residues in the C-terminal helix (hydrophobic stack) of the Gla domain.

EGF-like domains are small, rigid protein building blocks held together by a tight network of disulphide bonds. The EGF1 domain, consisting of residues 46–88, binds a single calcium ion close to the N-terminal end which is required for optimal affinity for TF [28]. There are indications that the presence of Ca^{2+} in the EGF1 domain locks the relative orientation of the Gla and EGF1 domains which would reduce the entropy loss upon TF binding [29]. Most of the hydrophobic interactions and hydrogen bonds in the FVIIa-TF complex are between the EGF1 domain and TF and the majority of the binding energy of the complex comes from this part of the interface (Fig 1A).

The EGF2 domain is tightly connected to the protease domain and together they form a structural unit bridged by a disulphide bond. The linker between the two EGF-like domains is flexible and acts as a hinge region between the two structural units, Gla/EGF1 and EGF2/protease domains. This notion is supported by the X-ray crystallographic structure of Gla-domainless FVIIa where the orientation of the EGF1 domain appears to be dictated by crystal packing, and a single residue, Gln^{88}, seems to act as the hinge [30]. The observed orientation probably represents one of a number of possibilities in solution. In another structure of Gla-domainless FVIIa, only the EGF2 and protease domains were well resolved suggesting a flexible arrangement of the EGF1 domain or disorder within the crystal [31]. Regardless of orientation and occupancy by TF, the EGF-like domains exhibit essentially identical conformations as expected considering their structural rigidity.

The protease domain structure is outlined in Figure 2. The core structure is similar to that of other trypsin-like serine proteases and consists of two halves, an N-terminal and a C-terminal β-barrel. His^{193}{57} and Asp^{242}{102} of the catalytic triad are located in the N-terminal barrel, while the active site Ser^{344}{195} is placed in the other subdomain. A comparison of X-ray crystallographic structures of trypsin and trypsinogen [32] shows that loops in the C-terminal β-barrel become ordered during trypsinogen activation (seen as markedly decreased crystallographic B factors). This prompted the definition of the activation domain which encompasses the N-terminal tail created upon endoproteolytic activation and the three so-called activation loops (Fig. 2). Insertion of the newly formed N terminus (Ile^{153}{16}) into the activation pocket to form a salt bridge with Asp^{343}{194} occurs spontaneously in most trypsin-like proteases and is required to define the active site region, i.e. to order the activation loops, mature the S1 pocket and generate the oxyanion hole. Zymogen FVII is converted to the activated form by proteolytic cleavage of the Arg^{152}{15}-Ile^{153}{16} peptide bond. The propensity of the N terminus in free FVIIa to become buried is low, rendering the protein zymogen-like. An exposed N terminus leads to a more rapid decrease in activity in experiments chemically modifying the α-amino group [12, 33]. FVIIa realizes its full catalytic power first when bound to TF, which allosterically promotes the N-terminal insertion [12], and TF binding activates FVIIa thereby creating optimal conditions for processing of the natural substrates, factors VII, IX and X. Apart from TF, Ca^{2+} also activates FVIIa albeit more modestly [34]. In Figure 2, the Ca^{2+} bound to the protease domain is shown as a purple sphere in the N-

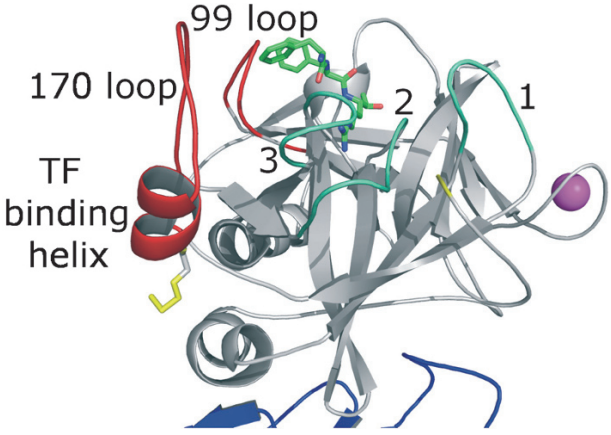

Figure 2. The protease domain of FVIIa (Protein Database entry code: 1dan.pdb) in cartoon representation (grey). Parts of the EGF2 domain are visible (blue). The TF-binding helix (306{164}–312{170}), the 170 loop (313{170A}–325{178}) and the 99 loop (233{93}–242{102}) are in red. The TF-interactive Met^{306}{164} is shown as a stick model in yellow. The activation loops 1 (285{142}–294{152}), 2 (331{184A}–342{193}) and 3 (365{216}–372{223}) are in cyan. The carbon atoms of the active site inhibitor FFR-cmk are in green and the Ca^{2+} ion is in purple.

terminal β-barrel. The presence of Ca^{2+} stimulates the enzymatic activity and, together with the other bound calcium ions, facilitates binding to TF [35]. This illustrates that apo-FVIIa is in a state of lower activity and in an equilibrium shifted further towards the zymogen-like conformation than Ca^{2+}-loaded FVIIa. In contrast, Zn^{2+} binding to the protease domain of FVIIa resulted in decreased activity. Theoretical methods identified two putative Zn^{2+}-binding sites in the vicinity of the Ca^{2+}-binding loop and suggested competition between the two ions [36]. X-ray crystallography has confirmed that Zn^{2+} indeed binds in the vicinity of the Ca^{2+} loop in the protease domain [37]. In recent years, a number of X-ray structures have illustrated the conformational plasticity of the protease domain of FVIIa in its catalytically competent conformation, i.e. with the N terminus buried in the activation pocket and an inhibitor occupying the active site. This is, in particular, observed in the vicinity of the S1 pocket [37, 38], in the first part of activation loop 3 and in the area of the TF-interactive helix and the 170 loop [18, 39]. The oxyanion hole and the rim of the S1 pocket may undergo minor conformational changes depending on the type of inhibitor bound. A structural comparison shows that not only the classical activation loop 3 (residues 365{216}–372{223}) is flexible in the substrate-binding cleft of FVIIa. Altogether, these observations indicate a high degree of flexibility in this region of FVIIa. When the inhibitor benzamidine is soaked out of FVIIa, the resulting structure reveals a slightly rotated TF-binding helix and a disordered 170 loop [39]. Thus there is a larger conformational flexibility in the absence of the inhibitor also in this region also. However, the N terminus remained buried in the activation pocket whether benzamidine was present or not suggesting that FVIIa did not return to the zymogen-like conformation when the inhibitor was soaked out.

Zymogen FVII has been crystallized in complex with an exosite-binding inhibitory peptide. The structure revealed a new registration of two β strands which considerably changed the TF-binding region and the macromolecular substrate exosite when compared to previous structures of FVIIa [40]. Subsequent analysis of the crystal packing showed intermolecular contacts at the site of the reregistration which makes it unlikely that the β sheet organization plays a role in FVIIa activation [41]. This was further substantiated by mutagenesis [42] and most recently by hydrogen exchange experiments on zymogen FVII and FVIIa in solution [43]. Nevertheless, locking the two β strands by an introduced disulphide bond in what was assumed to be the position in the active form of FVIIa resulted in variants with an apparently enhanced amidolytic activity accompanied by an altered specificity profile, but the intrinsic ability to activate the natural macromolecular substrate FX was not improved [44]. Detailed insights into the structure of uninhibited, free FVIIa are very much needed in order to reveal both the salient and subtle differences between the zymogen-like form and the enzymatically active (TF-bound) form of FVIIa.

Allosteric activation

A fraction of the FVII molecules in the blood are cleaved after Arg^{152}{15} and circulate in a form ready to assume the active conformation upon TF exposure. This patrolling function of FVIIa ensures rapid initiation of blood clotting upon tissue injury. At the same time, the preferred zymogen-like conformation of free FVIIa prevents untimely procoagulant activity. The allosteric activation of FVIIa by TF has been explored by a variety of techniques, e.g. site-directed mutagenesis and structural comparisons of TF-bound [18] and free FVIIa [30, 31], and reviewed [45, 46]. The alanine scanning mutagenesis [19] of 112 solvent-exposed residues identified the importance of Met^{306}{164} for activation by TF, and the X-ray structure of the complex confirmed its pivotal role [18]. The activation was indeed abolished when substituting with an Asp residue at this position [47]. Met^{306}{164} fits into a small surface cavity on TF and the interaction appears to induce an α-helical structure comprising residues 307{165}–312{170}, the TF-binding helix, that restricts the flexibility of the 170 loop. In the structure of free FVIIa, the α helix as well as the 170 loop was less ordered, suggesting an important impact of TF [18, 30]. The alanine scan also revealed exosites in the protease domain important for binding of the macromolecular substrates that are confined to the vicinity of the activation pocket. Recently, studies using hydrogen exhange monitored by mass spectroscopy [43] further elucidated the allosteric impact of TF on FVIIa. The hydrogen exchange kinetics of FVII and FVIIa were indistinguishable, indicating very similar solution structures. Upon TF binding, FVIIa underwent dramatic structural stabilization as indicated by decreased exchange rates distributed throughout the protease domain and in distant parts of the light chain. In an attempt to track the conformational changes induced by TF, exchange data were supplemented with molecular dynamics simulations [48]. This analysis suggested an allosteric activation mechanism in which TF stabilizes the environment of Met^{306}{164}, in particular the Leu^{305}{163}/Phe^{374}{225} pair, which in turn stabilizes activation loop 3, the S1 and S3 substrate binding sites,

the activation pocket and the N-terminal insertion. The replacement of Leu^{305}{163} with Val has been shown to give improved catalytic activity [49] supporting the hypothesis and the importance of the TF-interactive region.

Three regions of FVIIa are stabilized by TF [45]; the TF-binding region mentioned above (including the 170 loop) and, allosterically, the active site region and the macromolecular substrate exosite (encompassing the activation pocket) which determines physiological substrate specificity [50, 51]. This is why TF binding results in both an insertion of the N terminus into the activation pocket and a maturation of the active site. Interestingly, binding of an inhibitor in the active site of FVIIa increases the affinity for TF (stabilizes the TF-binding region) and promotes the insertion of the N terminus [33, 52, 53]. Analogously, the macromolecular substrate exosite is connected to the active site and TF-binding region [54, 55]. This demonstrates the bidirectional nature of the allosteric cross-talk.

Pharmacological mechanism of action of FVIIa

The total concentration of circulating FVII is 10 nM and only about 1 % of this is in the potentially active, cleaved FVIIa form. Keeping this in mind, one can anticipate that the administration of a typical dose of rFVIIa, around 100 μg/kg body weight which results in a circulating concentration of about 25 nM (or 250 times the endogenous FVIIa concentration), opens up an alternative mechanism of action. The affinity of FVIIa for the platelet membrane is characterized by an equilibrium binding constant of about 90 nM [56] or even as high as a couple of hundred nanomolar [E. Persson, unpublished data]. The high FVIIa concentration during haemostatic therapy makes it thermodynamically possible for a significant number of molecules to bind to the surface of activated platelets making platelet localization of an amount of FVIIa sufficient for haemostasis feasible during treatment with rFVIIa. FX has a higher affinity for the platelet membrane (binding constant around 30–50 nM, i.e. below the plasma concentration of FX of 140 nM). The colocalization of the enzyme FVIIa and its substrate FX allows for the pharmacological action of rFVIIa and forms the basis for its TF-independent mechanism of action (Figure 3).

Under haemophilic conditions, platelet-bound rFVIIa carries out the activation of FX normally done by the FIXa-FVIIIa complex. When added to 'normal' blood with intact functional levels of all factors, rFVIIa assists the tenase complex in the FXa generation process. In both cases, rFVIIa-catalysed FX activation provides the needed augmentation of the process and

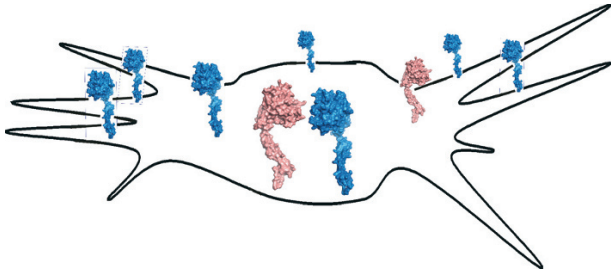

Figure 3. Schematic illustration of the colocalization of FVIIa and FX on the activated platelet surface during rFVIIa therapy. FX (blue) has an affinity for the platelet membrane that permits binding at the physiological concentration, whereas FVIIa (brown) only binds at pharmacological concentrations. The platelet surface brings the enzyme and the substrate together and serves as a template for (TF-independent) FVIIa-catalysed FX activation, a reaction that occurs extremely slowly in solution. Other surface molecules are omitted for clarity.

occurs on the surface of activated platelets. The thrombin that is subsequently generated by FXa not only cleaves fibrinogen to form a fibrin clot but also down-regulates fibrinolysis by activating the thrombin-activatable fibrinolysis inhibitor (TAFI). Thus the thrombin generation resulting from the addition of rFVIIa has both procoagulant and antifibrinolytic effects by promoting the formation of the fibrin clot, its structure [57] and its stability [58].

The limitations of FVIIa lie in its low platelet membrane affinity and low intrinsic activity, and the currently prevailing mechanism of action [59–61] suggests two obvious main routes towards an enhanced and more immediate pharmacological effect of FVIIa: the accumulation of a larger number of FVIIa molecules on the platelet surface by increasing the phospholipid affinity, or the enhancement of the intrinsic enzymatic activity of the individual FVIIa molecule. FVIIa variants with such improved properties have been constructed and are described in the following sections.

FVIIa analogues with increased membrane affinity

The major function of the Gla domain is to attach FVIIa to membranes, in other words to add binding energy to the interaction with membrane-associated TF. During treatment with rFVIIa, the task is changed to include mediation of platelet membrane binding. As mentioned above, the membrane affinity varies drastically between Gla domains of different proteins. An analysis of the correlation between amino acid sequence and membrane-binding properties suggested the importance of a few specific residues, namely those at positions 10, 32 and 33 in FVIIa (11, 33 and 34 in prothrombin numbering) [27]. Furthermore, the ω

loop of FVIIa is one amino acid shorter than in prothrombin. These observations prompted Nelsestuen and coworkers to construct a range of FVIIa mutants. The mutant P10Q-FVIIa showed a mere 2-fold enhancement in membrane binding affinity over FVIIa. A combination with K32E gave a further 25-fold enhancement [62], and this double mutant displayed a significantly improved procoagulant effect in haemophilic blood [63]. The analogue with the highest membrane affinity, P10Q/K32E/D33F/A34E-FVIIa, with a Tyr residue inserted at position 4, showed 150- to 300-fold improvement over FVIIa. The enhancement of membrane affinity of the FVIIa variants was related to an improvement in their coagulant activity of up to 40-fold depending on the assay method [64, 65]. An FVIIa variant based on this family is currently a clinical development candidate with Maxygen under the name MAXY-VII [66]. Except for the insertion at position 4, most of the mutations are in a region close to residue 32. This region is situated on the opposite side of the Gla domain relative to the common solvent-exposed hydrophobic patch (Phe^4, Leu^5 and Leu^8 in FVIIa) and quite far from the N-terminal end of the domain where the membrane contact is generally assumed to take place. X-ray and nuclear magnetic resonance (NMR) spectroscopy structures of the bovine prothrombin Gla domain in complex with Ca^{2+} and lysophosphatidylserine allowed the identification of critical interactions between the phospholipid and Gla [22]. In Figure 4, the Gla domain os FVIIa is shown in a cartoon model with mutated residues shown as sticks and lysophosphatidylserine modelled using a conformation found in the Gla domain of prothrombin. Molecular dynamics simulations provided structural and dynamic support for the role of the P10Q and K32E mutations in the improvement of the membrane contact [67]. A rotation of the Gly^{11} main chain occurring during the simulation results in a hydrogen bond with the Gln^{10} side chain and the appearance of a hydrogen bond between Glu^{32} and Gln^{10}. This forces the loop harbouring Arg^9 and Arg^{15} to shrink and thereby enhances the accessibility of the phospholipids to the Ca^{2+} ions.

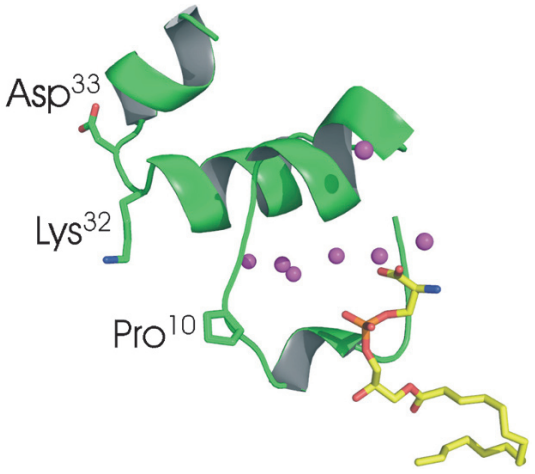

Figure 4. X-ray structure of the Gla domain of FVIIa (Protein Database entry code: 1dan.pdb) in complex with lysophosphatidylserine (yellow carbon atoms). The Ca^{2+} ions are in purple and side chains of interest are shown as stick models.

FVIIa analogues with a stabilized activation domain and N-terminal insertion

As mentioned above, a structurally well-defined activation domain and insertion of the protease domain N terminus are prerequisites for the activation of trypsin-like enzymes, and TF promotes this transition of FVIIa from a zymogen-like to an active conformation [12]. Therefore, in the attempts to generate FVIIa variants with elevated intrinsic enzymatic activity, mutations aimed at stabilizing the activation domain in the conformation that permits burial of the N terminus as well as mutations that should restrict the motional freedom of the N-terminal tail have been exploited. The sites and nature of the mutations are decided based on analyses and comparisons of the three-dimensional structures of FVIIa and its relatives and amino acid sequence alignments. One of the most active FVIIa variants, V158D/E296V/M298Q-FVIIa ($FVIIa_{DVQ}$, currently in clinical development by Novo Nordisk A/S under the name NN1731), combines these contributions, and its N terminus is very inaccessible to solvent (even slightly more buried than that of FVIIa in complex with soluble TF) [68, 69]. Figure 5 depicts the activation pockets of FVIIa and $FVIIa_{DVQ}$ with buried N termini. The introduced changes in $FVIIa_{DVQ}$ presumably promote generation of a strong hydrogen bond network fixing the N terminus in the activation pocket which may explain why the local, structural effect of the mutations is more profound than that allosterically induced by TF. However, the N terminus of an $FVIIa_{DVQ}$ molecule is presumably not buried in a different way than that of TF-bound or free FVIIa (when buried). Rather, the active conformation is favoured in $FVIIa_{DVQ}$ and the equilibrium between zymogen-like and active conformation is dramatically shifted towards the latter. As a consequence, $FVIIa_{DVQ}$ has a more mature active site than FVIIa and activates factor X about 30 times faster in solution as well as on the surface of activated platelets [68, 70]. This is apparently only, or at least predominantly, because of an increase in k_{cat}. The only marginally, if at all, reduced K_m of $FVIIa_{DVQ}$ for FX fits with the

observation that the affinity of the interaction between FVIIa and FX appears to be unaffected by the burial status of the protease domain N terminus [71]. The dramatically enhanced procoagulant or haemostatic potential of $FVIIa_{DVQ}$ and related FVIIa variants has also been demonstrated in haemophilic human plasma [72], in a murine model of haemophilia A [73] and, most recently, in whole blood from patients with severe haemophilia A [74, 75]. Of the individual substitutions in $FVIIa_{DVQ}$, only the M298Q mutation is able to enhance significantly the intrinsic activity, albeit to a more modest extent [68, 76]. However, the charged aspartic acid residue at position 158{21} is pivotal for high proteolytic activity (without it, amidolytic and proteolytic activities increase in parallel) [69] and removal of the charged residue in position 296{154} alleviates the electrostatic repulsion between the activation domain and the Ca^{2+}-binding loop (residues 210–220) [77]. Along with the enhanced activity of $FVIIa_{DVQ}$ and the other analogues comes an increased susceptibility to inhibition whether mediated by small active site inhibitors or by macromolecules such as antithrombin or tissue factor pathway inhibitor [68, 69, 76, 78, 79]. Not only glutamine but also lysine at position 298{156} can increase the intrinsic activity of FVIIa if in the correct environment [80]. The entire loop containing Glu^{296}{154} and Met^{298}{156} was replaced by that from FXa and unfavourable intramolecular contacts were removed. Interestingly, we observed an enhanced rate of FIX cleavage, but not of FX, indicating that it is possible to selectively improve macromolecular substrate processing.

An unrelated, activity-enhancing mutation in the vicinity is the replacement of Lys^{337}{188} by Ala [68] (see Fig. 5). It was originally hypothesized that the lysine residue, situated in activation loop 2, might repel the N terminus on its way into the activation pocket and that a charge elimination would facilitate N-terminal insertion. A threefold increase in intrinsic enzymatic activity was indeed obtained with the same absolute activity increase both on wild-type and $FVIIa_{DVQ}$ backgrounds, but the accessibility of the N terminus was unaltered. Thus the mechanism behind the activity enhancement of the K337A mutation remains elusive, but it is presumably related to its proximity to a catalytically important residue such as Asp^{338}{189}.

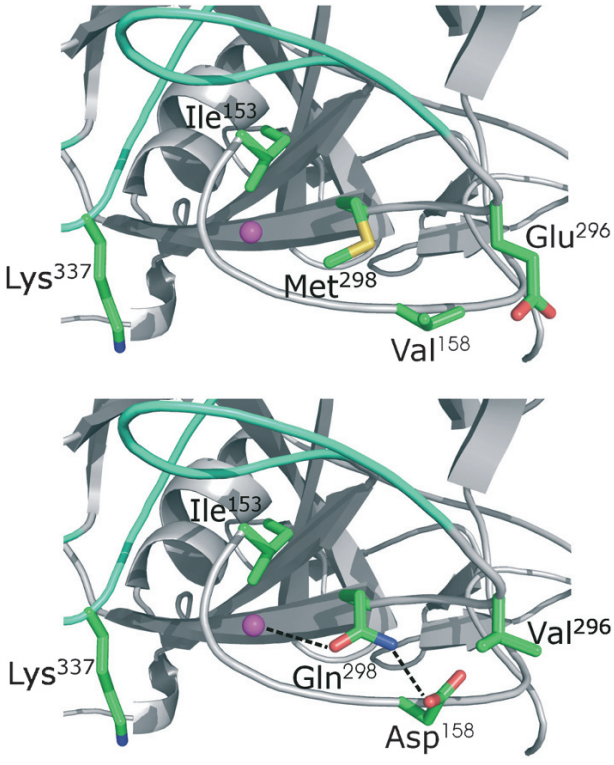

Figure 5. Close-up of the activation pockets in FVIIa (Protein Database entry code: 1dan.pdb) and in a model of $FVIIa_{DVQ}$. In the top panel, the side chains of Ile^{153}{16}, Val^{158}{21}, Glu^{296}{154}, Met^{298}{156} and Lys^{337}{188} are in stick models and a structurally conserved water molecule is shown as a magenta sphere. The N-terminal Ile^{153}{16} is buried in the activation pocket surrounded by the activation loops (in cyan). The water molecule, Ile^{153}{16}, Val^{158}{21}, Glu^{296}{154} and Met^{298}{156} all play a role in the, albeit poor, N-terminal insertion in FVIIa. In the bottom panel, a model of $FVIIa_{DVQ}$ is shown. A hydrogen bond network is presumably established between the water molecule and the introduced Gln and Asp side chains in position 298 and 158, respectively, promoting the burial of the N terminus.

FVIIa analogues with modifications affecting the 170 loop

Upon binding to FVIIa, as part of the stabilization of its active conformation, TF restricts the flexibility and alters the conformation of the 170 loop which is located between cysteines 310{168} and 329{182} and extends strategically from the cofactor-binding site towards the active site. The TF effect is perhaps best illustrated by the reduction of the susceptibility of the carbohydrate moiety on Asn^{322}{175} to deglycosylation [48] but it has also been inferred from hydrogen exchange experiments [43]. In analogy to the case with the activation domain and N-terminal insertion, mutational approaches were also designed to try to mimic this effect of TF in order to identify FVIIa variants with higher intrinsic activity. Leu^{305}{163} determines the relative orientation of the short, TF-interactive 307–312 α-helix and in turn influences the direction of the following long 170 loop. By mutating it to valine, a helix (and loop) orientation more similar to that of related and more active enzymes such as thrombin and factor Xa might be obtained. This has not been verified, but L305V-FVIIa displayed three times

higher intrinsic activity than FVIIa [49]. An attempt to restrict the flexibility of the 170 loop more directly was made by introducing the S314E and F374Y mutations to anchor the loop to the body of the protease domain [78]. When introduced individually, the former mutation gave a 2.5-fold increase in the intrinsic activity, whereas the latter mutation was silent. When combined with the L305V and K337A mutations, giving $FVIIa_{VEAY}$, the resulting molecule exhibited more than 20-fold enhanced catalytic efficiency as measured with a peptide substrate (4-fold higher k_{cat} and 5.5-fold lower K_m values) and was capable of activating factor X about 10 times faster (exclusively k_{cat} improvement) than FVIIa [78]. Figure 6 shows a close-up of the TF-binding helix and the 170 loop in FVIIa and a model of $FVIIa_{VEAY}$. In $FVIIa_{VEAY}$, hydrogen bonds have been suggested between the side chains of the Tyr residue in position 374 and Gln^{313}{170A} and between the Glu residue in position 314 and His^{373}{224}. Together they may tighten the connection of the TF binding helix and the rest of the protease domain, resulting in general structural stabilization. This notion is supported by the experimental observation that the enhanced activity of $FVIIa_{VEAY}$ is mediated by maturation of the S1 subsite which results in an increased k_{cat} value and, for substrates whose binding affinity is dictated by interactions close to the scissile bond, a reduced K_m value. In contrast to $FVIIa_{DVQ}$, there is no evidence of a shift in the equilibrium towards the active FVIIa conformation in $FVIIa_{VEAY}$ because the N terminus is left as exposed as in FVIIa.

Along another avenue, variants of FVIIa with enhanced intrinsic activity were made by truncating the 170 loop or replacing it with that from trypsin. The rationale is that the loop in FVIIa is five residues longer and therefore more flexible and counterproductive than the corresponding loop in other members of the trypsin family. The grafted variant displayed increased activity but with an accompanying specificity change and without evidence of N-terminal insertion [81], whereas the simple truncation had a marginal, if any, effect on factor X activation. This demonstrates that the influence of the 170 loop on the intrinsic FVIIa activity is more complex and not only dictated by loop length. Additional grafting of the so-called 99 loop from trypsin had a slight opposite effect on the ability to activate factor X although it further improved the amidolytic activity [82].

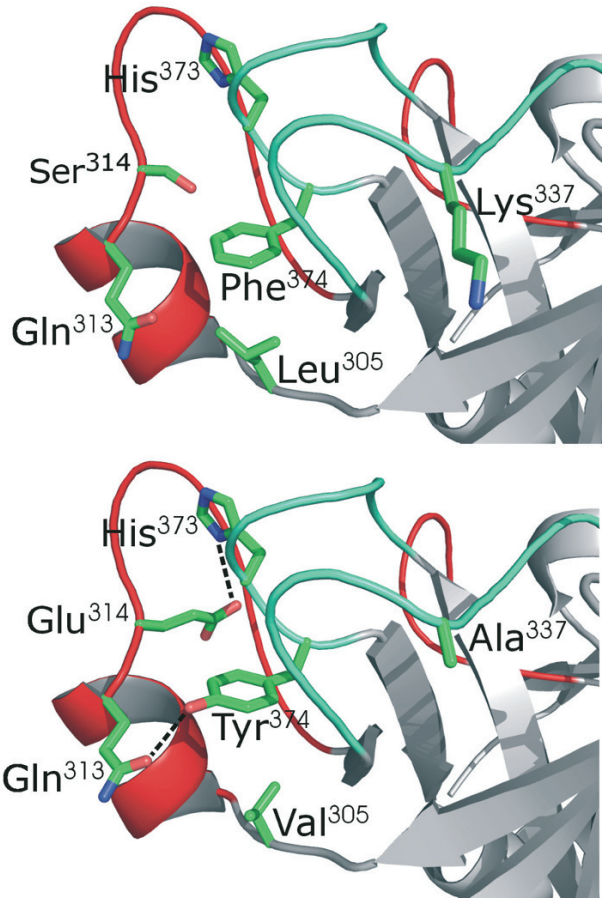

Figure 6. Close-up of the TF-binding helix and the 170 loop in FVIIa (Protein Database entry code: 1dan.pdb) and in a model of $FVIIa_{VEAY}$. Activation loops 2 and 3 (in cyan) interact with the TF-binding helix and the 170 loop. In the top panel, the side chains of Lys^{337}{188}, His^{373}{224}, Phe^{374}{225}, Leu^{305}{163}, Gln^{313}{170A} and Ser^{314}{170B} are in stick models. Phe^{374}{225}, Leu^{305}{163} and Ser^{314}{170B} form a cluster in the interaction region between the TF-binding helix and the body of FVIIa. In the bottom panel, a model of $FVIIa_{VEAY}$ is shown. Hydrogen bonds have been suggested between the Tyr side chain in position 374 and Gln^{313}{170A}, and between the Glu side chain in position 314 and His^{373}{224} which may tighten the connection of the TF binding helix to the rest of the protease domain.

Future perspectives

Even though much knowledge has accumulated, many details of the TF-induced structural changes in FVIIa and the consequent activity increase remain elusive. The solution of the structure of free and uninhibited FVIIa, either by high-resolution X-ray crystallographic or solution techniques, would be a giant leap forward in our understanding of this pivotal biological event, and future efforts will certainly be focused on this endeavour. The identification and development of new, superior FVIIa analogues for clinical use has great potential. The crucial challenge might be to balance haemostatic efficacy and safety. An insurance against adverse events while improving the biological activity is the preservation of FVIIa specificity. It will be exciting to follow the ground-breaking FVIIa analogues, MAXY-VII and NN1731, on their journey towards approval and we will hopefully see new

analogues approaching and entering clinical development in the future.

1 Davie, E. W., Fujikawa, K. and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. Biochemistry 30, 10363–10370.
2 Kalafatis, M., Swords, N. A., Rand, M. D. and Mann, K. G. (1994) Membrane-dependent reactions in blood coagulation: role of the vitamin K-dependent enzyme complexes. Biochim. Biophys. Acta 1227, 113–129.
3 Monroe, D. M. and Key, N. S. (2007) The tissue factor-factor VIIa complex: procoagulant activity, regulation, and multitasking. J. Thromb. Haemost. 5, 1097–1105.
4 Hoffman, M., Colina, C. M., McDonald, A. G., Arepally, G. M., Pedersen, L. and Monroe, D. M. (2007) Tissue factor around dermal vessels has bound factor VII in the absence of injury. J. Thromb. Haemost. 5, 1403–1408.
5 Monroe, D. M., Hoffman, M. and Roberts, H. R. (2002) Platelets and thrombin generation. Arterioscler. Thromb. Vasc. Biol. 22, 1381–1389.
6 Monroe, D. M. and Hoffman, M. (2006) What does it take to make the perfect clot? Arterioscler. Thromb. Vasc. Biol. 26, 41–48.
7 Rao, L. V. M. and Pendurthi, U. R. (2005) Tissue factor-factor VIIa signaling. Arterioscler. Thromb. Vasc. Biol. 25, 47–56.
8 Furie, B. and Furie, B. C. (1988) The molecular basis of blood coagulation. Cell 53, 505–518.
9 Hansson, K. and Stenflo, J. (2005) Post-translational modifications in proteins involved in blood coagulation. J. Thromb. Haemost. 3, 2633–2648.
10 McCallum, C. D., Hapak, R. C., Neuenschwander, P. F., Morrissey, J. H. and Johnson, A. E. (1996) The location of the active site of blood coagulation factor VIIa above the membrane surface and its reorientation upon association with tissue factor. J. Biol. Chem. 271, 28168–28175.
11 Khan, A. R. and James, M. N. G. (1998) Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci. 7, 815–836.
12 Higashi, S., Nishimura, H., Aita, K. and Iwanaga, S. (1994) Identification of regions of bovine factor VII essential for binding to tissue factor. J. Biol. Chem. 269, 18891–18898.
13 Silverberg, S. A., Nemerson, Y. and Zur, M. (1977) Kinetics of the activation of bovine coagulation factor X by components of the extrinsic pathway. J. Biol. Chem. 252, 8481–8488.
14 Lavigne-Lissalde, G., Schved, J.-F., Granier, C. and Villard, S. (2005) Anti-factor VIII antibodies: a 2005 update. Thromb. Haemost. 94, 760–769.
15 Roberts, H. R., Monroe, D. M. and White, G. C. (2004) The use of recombinant factor VIIa in the treatment of bleeding disorders. Blood 104, 3858–3864.
16 Franchini, M., Zaffanello, M. and Veneri, D. (2005) Recombinant factor VIIa: an update on its clinical use. Thromb. Haemost. 93, 1027–1035.
17 Siddiqui, M. A. A. and Scott, L. J. (2005) Recombinant factor VIIa (eptacog alfa): a review of its use in congenital or acquired haemophilia and other congenital bleeding disorders. Drugs 65, 1161–1177.
18 Banner, D. W., D'Arcy, A., Chène, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y. and Kirchhofer, D. (1996) The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. Nature 380, 41–46.
19 Dickinson, C. D., Kelly, C. R. and Ruf, W. (1996) Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa. Proc. Natl. Acad. Sci. USA 93, 14379–14384.
20 O'Hara, P. J., Grant, F. J., Haldeman, B. A., Gray, C. L., Insley, M. Y., Hagen, F. S. and Murray, M. J. (1987) Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. Proc. Natl. Acad. Sci. USA 84, 5158–5162.
21 Stenflo, J., Fernlund, P., Egan, W. and Roepstorff, P. (1974) Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proc. Natl. Acad. Sci. USA 71, 2730–2733.
22 Huang, M., Rigby, M. C., Morelly, X., Grant, M., Huang, G., Furie, B., Seaton, B. and Furie, B. C. (2003) Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. Nat. Struct. Biol. 10, 751–756.
23 Sunnerhagen, M., Forsén, S., Hoffrén, A.-M., Drakenberg, T., Teleman, O. and Stenflo, J. (1995) Structure of the Ca^{2+}-free Gla domain sheds light on membrane binding of blood coagulation proteins. Nat. Struct. Biol. 2, 504–509.
24 Persson, E. and Petersen, L. C. (1995) Structurally and functionally distinct Ca^{2+} binding sites in the γ-carboxyglutamic acid-containing domain of factor VIIa. Eur. J. Biochem. 234, 293–300.
25 Freedman, S. J., Blostein, M. D., Baleja, J. D., Jacobs, M., Furie, B. C. and Furie, B. (1996) Identification of the phospholipid binding site in the vitamin K-dependent blood coagulation protein factor IX. J. Biol. Chem. 271, 16227–16236.
26 Grant, M. A., Baikeev, R. F., Gilbert, G. E. and Rigby, A. C. (2004) Lysine 5 and phenylalanine 9 of factor IX ω-loop interact with phosphatidylserine in a membrane mimic environment. Biochemistry 43, 15367–15378.
27 McDonald, J. F., Shah, A. M., Schwalbe, R. A., Kisiel, W., Dahlbäck, B. and Nelsestuen, G. L. (1997) Comparison of naturally occurring vitamin K-dependent proteins: correlation of amino acid sequences and membrane binding properties suggests a membrane contact site. Biochemistry 36, 5120–5127.
28 Persson, E., Olsen, O. H., Østergaard, A. and Nielsen, L. S. (1997) Ca^{2+} binding to the first epidermal growth factor-like domain of factor VIIa increases amidolytic activity and tissue factor affinity. J. Biol. Chem. 272, 19919–19924.
29 Sunnerhagen, M., Olah, G. A., Stenflo, J., Forsén, S., Drakenberg, T. and Trewhella, J. (1996) The relative orientation of Gla and EGF domains in coagulation factor X is altered by Ca^{2+} binding to the first EGF domain: a combined NMR-small angle x-ray scattering study. Biochemistry 35, 11547–11559.
30 Pike, A. C. W., Brzozowski, A. M., Roberts, S. M., Olsen, O. H. and Persson, E. (1999) Structure of human factor VIIa and its implications for the triggering of blood coagulation. Proc. Natl. Acad. Sci. USA 96, 8925–8930.
31 Kemball-Cook, G., Johnson, D. J. D., Tuddenham, E. G. D. and Harlos, K. (1999) Crystal structure of active site-inhibited human coagulation factor VIIa (des-Gla). J. Struct. Biol. 127, 213–223.
32 Fehlhammer, H., Bode, W. and Huber, R. (1977) Crystal structure of bovine trypsinogen at 1.8 Å resolution. II. Crystallographic refinement, refined crystal structure and comparison with bovine trypsin. J. Mol. Biol. 111, 415–438.
33 Higashi, S., Matsumoto, N. and Iwanaga, S. (1996) Molecular mechanism of tissue factor-mediated acceleration of factor VIIa activity. J. Biol. Chem. 271, 26569–26574.
34 Wildgoose, P., Foster, D., Schiødt, J., Wiberg, F. C., Birktoft, J. J. and Petersen, L. C. (1993) Identification of a calcium binding site in the protease domain of human blood coagulation factor. VII. Evidence for its role in factor VII-tissue factor interaction. Biochemistry 32, 114–119.
35 Neuenschwander, P. F. and Morrissey, J. H. (1994) Roles of the membrane-interactive regions of factor VIIa and tissue factor. J. Biol. Chem. 269, 8007–8013.
36 Petersen, L. C., Olsen, O. H., Nielsen, L. S., Freskgård, P.-O. and Persson, E. (2000) Binding of Zn^{2+} to a Ca^{2+} loop allosterically attenuates the activity of factor VIIa and reduces its affinity for tissue factor. Protein Sci. 9, 859–866.
37 Bajaj, S. P., Schmidt, A. E., Agah, S., Bajaj, M. S. and Padmanabhan, K. (2006) High resolution structures of *p*-aminobenzamidine- and benzamidine-VIIa/soluble tissue factor: unpredicted conformation of the 192–193 peptide bond and mapping of Ca^{2+}, Mg^{2+}, Na^{+}, and Zn^{2+} sites in factor VIIa. J. Biol. Chem. 281, 24873–24888.

38 Groebke, Zbinden, K., Obst-Sander, U., Hilpert, K., Kühne, H., Banner, D. W., Böhm, H.-J., Stahl, M., Ackermann, J., Alig, L., Weber, L., Wessel, H. P., Riederer, M. A., Tschopp, T. B. and Lavé, T. (2005) Selective and orally bioavailable phenylglycine tissue factor/factor VIIa inhibitors. Bioorg. Med. Chem. Lett. 15, 5344–5352.
39 Sichler, K., Banner, D. W., D'Arcy, A., Hopfner, K.-P., Huber, R., Bode, W., Kresse, G.-B., Kopetzki, E. and Brandstetter, H. (2002) Crystal structures of uninhibited factor VIIa link its cofactor and substrate-assisted activation to specific interactions. J. Mol. Biol. 322, 591–603.
40 Eigenbrot, C., Kirchhofer, D., Dennis, M. S., Santell, L., Lazarus, R. A., Stamos, J. and Ultsch, M. H. (2001) The factor VII zymogen structure reveals reregistration of β strands during activation. Structure 9, 627–636.
41 Perera, L. and Pedersen, L. G. (2005) A reconsideration of the evidence for structural reorganization in FVII zymogen. J. Thromb. Haemost. 3, 1543–1545.
42 Olsen, O. H., Nielsen, P. F. and Persson, E. (2004) Prevention of β strand movement into a zymogen-like position does not confer higher activity to coagulation factor VIIa. Biochemistry 43, 14096–14103.
43 Rand, K. D., Jørgensen, T. J. D., Olsen, O. H., Persson, E., Jensen, O. N., Stennicke, H. R. and Andersen, M. D. (2006) Allosteric activation of coagulation factor VIIa visualized by hydrogen exchange. J. Biol. Chem. 281, 23018–23024.
44 Maun, H. R., Eigenbrot, C., Raab, H., Arnott, D., Phu, L., Bullens, S. and Lazarus, R. A. (2005) Disulfide locked variants of factor VIIa with a restricted β-strand conformation have enhanced enzymatic activity. Protein Sci. 14, 1171–1180.
45 Ruf, W. and Dickinson, C. D. (1998) Allosteric regulation of the cofactor-dependent serine protease coagulation factor VIIa. Trends Cardiovasc. Med. 8, 350–356.
46 Eigenbrot, C. and Kirchhofer, D. (2002) New insight into how tissue factor allosterically regulates factor VIIa. Trends Cardiovasc. Med. 12, 19–26.
47 Persson, E., Nielsen, L. S. and Olsen, O. H. (2001) Substitution of aspartic acid for methionine-306 in factor VIIa abolishes the allosteric linkage between the active site and the binding interface with tissue factor. Biochemistry 40, 3251–3256.
48 Olsen, O. H., Rand, K. D., Østergaard, H. and Persson, E. (2007) A combined structural dynamics approach identifies a putative switch in factor VIIa employed by tissue factor to initiate blood coagulation. Protein Sci. 16, 671–682.
49 Persson, E., Bak, H. and Olsen, O. H. (2001) Substitution of valine for leucine 305 in factor VIIa increases the intrinsic enzymatic activity. J. Biol. Chem. 276, 29195–29199.
50 Shobe, J., Dickinson, C. D., Edgington, T. S. and Ruf, W. (1999) Macromolecular substrate affinity for the tissue factor-factor VIIa complex is independent of scissile bond docking. J. Biol. Chem. 274, 24171–24175.
51 Baugh, R. J., Dickinson, C. D., Ruf, W. and Krishnaswamy, S. (2000) Exosite interactions determine the affinity of factor X for the extrinsic Xase complex. J. Biol. Chem. 275, 28826–28833.
52 Sørensen, B. B., Persson, E., Freskgård, P.-O., Kjalke, M., Ezban, M., Williams, T. and Rao, L. V. M. (1997) Incorporation of an active site inhibitor in factor VIIa alters the affinity for tissue factor. J. Biol. Chem. 272, 11863–11868.
53 Dickinson, C. D. and Ruf, W. (1997) Active site modification of factor VIIa affects interactions of the protease domain with tissue factor. J. Biol. Chem. 272, 19875–19879.
54 Dickinson, C. D., Shobe, J. and Ruf, W. (1998) Influence of cofactor binding and active site occupancy on the conformation of the macromolecular substrate exosite of factor VIIa. J. Mol. Biol. 277, 959–971.
55 Shobe, J., Dickinson, C. D. and Ruf, W. (1999) Regulation of the catalytic function of coagulation factor VIIa by a conformational linkage of surface residue Glu 154 to the active site. Biochemistry 38, 2745–2751.
56 Monroe, D. M., Hoffman, M., Oliver, J. A. and Roberts, H. R. (1997) Platelet activity of high-dose factor VIIa is independent of tissue factor. Br. J. Haematol. 99, 542–547.
57 He, S., Blombäck, M., Jacobsson, Ekman, G. and Hedner, U. (2003) The role of recombinant factor VIIa (FVIIa) in fibrin structure in the absence of FVIII/FIX. J. Thromb. Haemost. 1, 1215–1219.
58 Lisman, T., Mosnier, L. O., Lambert, T., Mauser-Bunschoten, E. P., Meijers, J. C. M., Nieuwenhuis, H. K. and de Groot, P. G. (2002) Inhibition of fibrinolysis by recombinant factor VIIa in plasma from patients with severe hemophilia A. Blood 99, 175–179.
59 Monroe, D. M., Hoffman, M., Oliver, J. A. and Roberts, H. R. (1998) A possible mechanism of action of activated factor VII independent of tissue factor. Blood Coagul. Fibrinolysis 9 (Suppl. 1), S15–S20.
60 Hoffman, M., Monroe, D. M. and Roberts, H. R. (1998) Activated factor VII activates factors IX and X on the surface of activated platelets: thoughts on the mechanism of action of high-dose activated factor VII. Blood Coagul. Fibrinolysis 9 (Suppl. 1), S61–S65.
61 Hedner, U. (2006) Mechanism of action, development and clinical experience of recombinant FVIIa. J. Biotechnol. 124, 747–757.
62 Shah, A. M., Kisiel, W., Foster, D. C. and Nelsestuen, G. L. (1998) Manipulation of the membrane binding site of vitamin K-dependent proteins: enhanced biological function of human factor VII. Proc. Natl. Acad. Sci. USA 95, 4229–4234.
63 Henderson, N., Key, N. S., Christie, B., Kisiel, W., Foster, D. and Nelsestuen, G. L. (2002) Response of factor VIII and IX-deficient blood to wild type and high membrane affinity mutant factor VIIa in an *in vitro* whole blood clotting assay: possible correlation to clinical outcome. Thromb. Haemost. 88, 98–103.
64 Nelsestuen, G. L., Stone, M., Martinez, M. B., Harvey, S. B., Foster, D. and Kisiel, W. (2001) Elevated function of blood clotting factor VIIa mutants that have enhanced affinity for membranes. J. Biol. Chem. 276, 39825–39831.
65 Harvey, S. B., Stone, M. D., Martinez, M. B. and Nelsestuen, G. L. (2003) Mutagenesis of the γ-carboxyglutamic acid domain of human factor VII to generate maximum enhancement of the membrane contact site. J. Biol. Chem. 278, 8363–8369.
66 Bornæs, C., Jensen, R. B., Röpke, M., Breinholt, V. M., Nygaard, F. B., Halkier, T., Andersen, K. V., Kisiel, W. and Haaning, J. (2007) Improved procoagulant and pharmacokinetic properties of two novel recombinant human factor VIIa variants prepared by directed molecular evolution and rational design. J. Thromb. Haemost. 5 (Suppl. 2), abstract O-W-038.
67 Taboureau, O. and Olsen, O. H. (2007) Computational study of coagulation factor VIIa's affinity for phospholipid membranes. Eur. Biophys. J. 36, 133–144.
68 Persson, E., Kjalke, M. and Olsen, O. H. (2001) Rational design of coagulation factor VIIa variants with substantially increased intrinsic activity. Proc. Natl. Acad. Sci. USA 98, 13583–13588.
69 Petrovan, R. J. and Ruf, W. (2002) Role of zymogenicity-determining residues of coagulation factor VII/VIIa in cofactor interaction and macromolecular substrate recognition. Biochemistry 41, 9302–9309.
70 Allen, G. A., Persson, E., Campbell, R. A., Ezban, M., Hedner, U. and Wolberg, A. S. (2007) A variant of recombinant factor VIIa with enhanced procoagulant and antifibrinolytic activities in an *in vitro* model of hemophilia. Arterioscler. Thromb. Vasc. Biol. 27, 683–689.
71 Persson, E. (2006) Macromolecular substrate affinity for free factor VIIa is independent of a buried protease domain N-terminus. Biochem. Biophys. Res. Comm. 341, 28–32.
72 Lisman, T., De Groot, P. G., Lambert, T., Røjkjær, R. and Persson, E. (2003) Enhanced *in vitro* procoagulant and antifibrinolytic potential of superactive variants of recombinant factor VIIa in severe hemophilia A. J. Thromb. Haemost. 1, 2175–2178.

73 Tranholm, M., Kristensen, K., Kristensen, A. T., Pyke, C., Røjkjær, R. and Persson, E. (2003) Improved hemostasis with superactive analogs of factor VIIa in a mouse model of hemophilia A. Blood 102, 3615–3620.
74 Sørensen, B., Persson, E. and Ingerslev, J. (2007) Factor VIIa analogue (V158D/E296V/M298Q-FVIIa) normalises clot formation in whole blood from patients with severe haemophilia A. Br. J. Haematol. 137, 158–165.
75 Brophy, D. F., Martin, E. J., Nolte, M. E., Kuhn, J. G. and Carr, Jr, M. E. (2007) Effect of recombinant factor VIIa variant (NN1731) on platelet function, clot structure and force onset time in whole blood from healthy volunteers and haemophilia patients. Haemophilia 13, 533–541.
76 Petrovan, R. J. and Ruf, W. (2001) Residue Met^{156} contributes to the labile enzyme conformation of coagulation factor VIIa. J. Biol. Chem. 276, 6616–6620.
77 Persson, E. and Olsen, O. H. (2002) Assignment of molecular properties of a superactive coagulation factor VIIa variant to individual amino acid changes. Eur. J. Biochem. 269, 5950–5955.
78 Persson, E., Bak, H., Østergaard, A. and Olsen, O. H. (2004) Augmented intrinsic activity of factor VIIa by replacement of residues 305, 314, 337 and 374: evidence of two unique mutational mechanisms of activity enhancement. Biochem. J. 379, 497–503.
79 Ghosh, S., Ezban, M., Persson, E., Pendurthi, U., Hedner, U. and Rao, L. V. M. (2007) Activity and regulation of factor VIIa analogs with increased potency at the endothelial cell surface. J. Thromb. Haemost. 5, 336–346.
80 Bjelke, J. R., Persson, E., Rasmussen, H. B., Kragelund, B. B. and Olsen, O. H. (2007) A loop of coagulation factor VIIa influencing macromolecular substrate specificity. FEBS Lett. 581, 71–76.
81 Soejima, K., Mizuguchi, J., Yuguchi, M., Nakagaki, T., Higashi, S. and Iwanaga, S. (2001) Factor VIIa modified in the 170 loop shows enhanced catalytic activity but does not change the zymogen-like property. J. Biol. Chem. 276, 17229–17235.
82 Soejima, K., Yuguchi, M., Mizuguchi, J., Tomokiyo, K., Nakashima, T., Nakagaki, T. and Iwanaga, S. (2002) The 99 and 170 loop-modified factor VIIa mutants show enhanced catalytic activity without tissue factor. J. Biol. Chem. 277, 49027–49035.