Prevention of β Strand Movement into a Zymogen-like Position Does Not Confer Higher Activity to Coagulation Factor VIIa

Ole H. Olsen,*,‡ Per F. Nielsen,§ and Egon Persson‡

Haemostasis Biochemistry, Novo Nordisk A/S, DK-2760 Måløv, Denmark, and Protein Science, Novo Nordisk A/S, DK-2880 Bagsværd, Denmark

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ABSTRACT: Coagulation factor VIIa (FVIIa) belongs to the chymotrypsin family of S1 peptidases, whose members require the cleavage of at least one internal peptide bond to attain an active conformation. FVIIa also requires association with tissue factor (TF) to attain full catalytic competency. Without this, FVIIa has very low activity toward peptide and physiologic substrates. Reregistration of β strands has been suggested to play a part in the activation of FVII, and their positioning is possibly important for the active conformation of FVIIa. To scrutinize this hypothesis, we have designed FVIIa variants which prevent β strand movement and lock FVIIa in the alleged active conformation. The V299M mutation, alone or combined with the L280I mutation, was introduced to alter the first of two Leu-X-Val motifs in β strand B2 and thereby prevent reregistration. Along the same line, C164V/V299C-FVIIa has a new disulfide which would keep β strand B2 in the registration of active FVIIa. The amidolytic and proteolytic activities of V299M-, L280I/V299M-, and C164V/V299C-FVIIa were indistinguishable from or lower than those of wild-type FVIIa, and none of the mutants displayed an altered exposure of the N-terminal amino group of the protease domain. Moreover, the affinities of mutant and native FVIIa for TF increased to a similar extent upon incorporation of an active site inhibitor, and the enzymatic activities were equally stimulated by TF. In conclusion, we found no evidence that the mutants were in a more active state than native FVIIa. Thus, the proposed β strand reregistration, if part of the regulatory mechanism governing FVIIa activity, apparently does not suffice for the transformation of FVIIa into an enzymatically active conformation. Our data raise the possibility that the structural differences between enzymatically latent (zymogen-like) and active FVIIa resemble those between trypsinogen and trypsin.

After vascular damage, factor VIIa (FVIIa)[1] associates with tissue factor (TF) to initiate the blood coagulation cascade (*1*). This complex formation is required to render FVIIa biologically active by dramatically enhancing the catalytic efficiency (*2*), which is readily measured as an increased rate of peptidic substrate hydrolysis (*3*). The allosteric mechanism by which TF stimulates the activity of FVIIa is under intense investigation.

FVIIa belongs to the chymotrypsin family of serine proteases, requiring specific endoproteolytic processing to attain the active conformation. Following zymogen activation, the amino terminus of the protease domain (Ile-153)[2] typically enters the so-called activation pocket to form a salt bridge with Asp-343. The activation pocket is part of the activation domain which has been shown to become ordered during trypsinogen activation (decreased crystallographic *B* factors) (*4*).The conformational change observed by X-ray crystallography when comparing trypsinogen to trypsin reveals a maturation of the S1 pocket as well as generation of the oxyanion hole that is important for catalytic competency (*4*, *5*). The insertion of the N-terminal amino group also protects it from chemical modification. However, the amino terminus of the protease domain of free FVIIa is still susceptible to chemical modification, indicative of retained solvent accessibility and a zymogen-like conformation. Binding of TF stabilizes the insertion of the N-terminus and promotes the active conformation (*6*). Thus, FVII is incompletely converted from zymogen to enzyme upon cleavage after Arg-152.

Considerable progress derived from functional and mutational studies has helped elucidate details of the allosteric regulation of FVIIa (*7*). The findings suggest that the regulation is not mediated by a simple, single conformational transition. The inherent flexibility of the activation region is employed to allosterically regulate the protease activity. It appears that three regions of the FVIIa protease domain are important for the allosteric regulation and function (*7*), i.e., the TF binding site, the active site region, and the macromolecular substrate exosite which also comprises the activation pocket (Figure 1A). Cross talk, in the form of

* To whom correspondence should be addressed: Haemostasis Biochemistry, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark. Fax: +45 44434547. Telephone: +45 44434511. E-mail: oho@novonordisk.com.

‡ Haemostasis Biochemistry.

§ Protein Science.

[1] Abbreviations: FVII(a), coagulation factor VII(a); FX(a), coagulation factor X(a); SPR, surface plasmon resonance; sTF, soluble tissue factor (residues 1−219); TF, tissue factor.

[2] Chymotrypsinogen numbering is as follows: Arg-152, 15; Ile-153, 16; Lys-157, 20; Cys-159, 22; Pro-160, 23; Glu-163, 26; Cys-164, 27; Pro-165, 28; Trp-166, 29; Leu-280, 137; Ala-294, 152; Glu-296, 154; Leu-297, 155; Met-298, 156; Val-299, 157; Leu-300, 158; Val-302, 160; Arg-304, 162; Met-306, 164; Gln-313, 170A; Asp-343, 194; Trp-356, 207; Tyr-377, 228.

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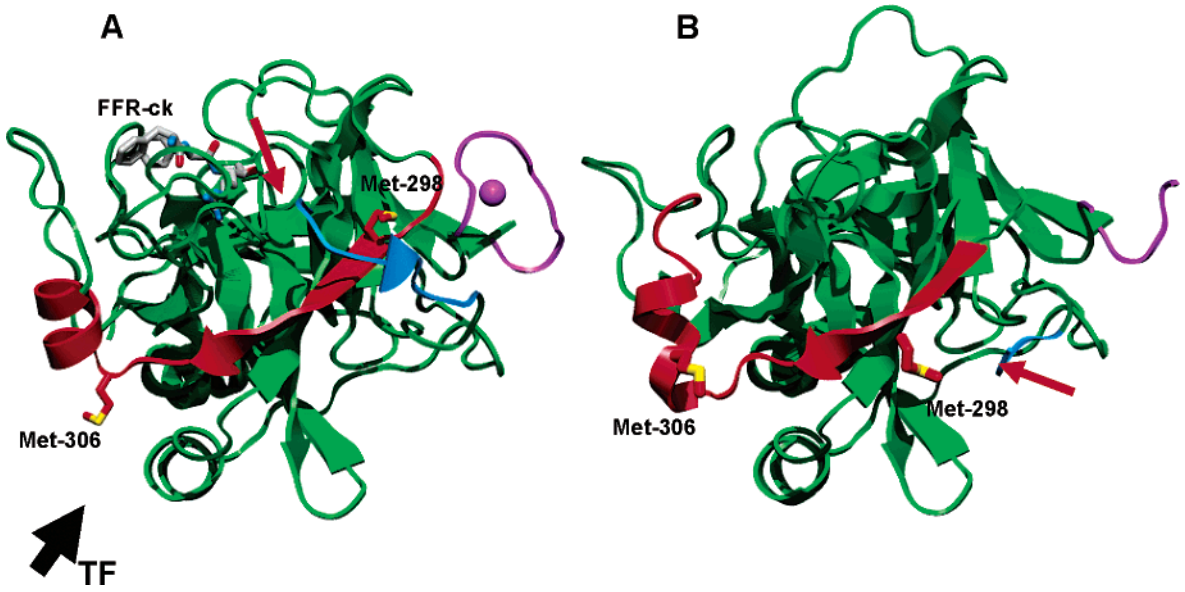

FIGURE 1: Protease domain of the active FVIIa conformation (A) (PDB entry 1dan) and zymogen FVII conformation (B) (PDB entry 1bju). Residues 294−313 subjected to reregistration are colored red. The side chain of Met-298 is shifted from panel A to B. In panel A, the calcium loop and the calcium ion are colored magenta. This part of the zymogen structure is poorly resolved in the structure in panel B. The N-termini are colored blue (red arrows). In panel A, the covalently bound inhibitor FFR-ck is shown as a stick model in light green and the black arrow marked TF indicates the direction in which the cofactor tentatively approaches and binds the protease domain. The three regions of the FVIIa protease domain important for function are the TF binding site (the region in the vicinity of Met-306), the active site region (the region close to the FFR-ck inhibitor), and the macromolecular substrate exosite (the region in the vicinity of Met-298). These regions communicate along unknown paths in response to binding of an allosteric regulator.

allosteric signals, is transmitted between these regions. Alanine scanning of FVIIa has identified, by loss of function, residues in the protease domain that are important for TF binding and catalytic function (*8*). It has also served as an inspiration for site-specific mutagenesis efforts aimed at elucidating the nature of the allostery or creating FVIIa analogues with enhanced activity (*9*−*15*). These gain-of-function variants have provided additional insights into the allosteric regulation. In contrast, no clues are offered by the three-dimensional structure of the protease domain of free FVIIa because it is, apart from certain loop regions, virtually identical to that of thrombin and other constitutively active serine proteases. In addition, the available structures of free (*16*−*18*) and TF-bound FVIIa (*19*, *20*) are very similar, perhaps due to the presence of an active site inhibitor in all structures. In ref *18*, a structure without an inhibitor in the active site was obtained by removing the inhibitor from a preformed crystal. However, the possibility that there are restrictions in the allowed conformational adjustments caused by the existing crystal cannot be ruled out. A structure of noninhibited FVIIa, formed in the absence of an inhibitor, is needed to reveal the potential structural differences between the zymogen-like and enzymatically active conformations of FVIIa, especially considering that the active site inhibitor exerts allosteric effects manifested as an influence on the affinity for TF (*21*, *22*). Nonetheless, information possibly pertaining to the zymogen-like conformation of free FVIIa has been provided by the solution of the crystal structure of zymogen FVII (*23*). The zymogen, however, was crystallized in complex with an exosite-binding inhibitory peptide. The structure reveals a new registration of two β strands (Figure 1), resulting in large differences in the region binding TF as well as in the macromolecular substrate exosite region compared to previous structures of FVIIa. TF binding is hypothesized to reorganize the macromolecular substrate exosite, resulting in the insertion of the N-terminal Ile-153 into the activation pocket, and to induce the shift of a β strand by three amino acid residues to attain the active FVIIa conformation (*23*). The FVII(a) sequence contains a duplicated Leu-X-Val repeat in positions 297−302 which is believed to facilitate β strand reregistration.

In the chymotrypsin family, comprising proteases with closely related enzyme structures, at present two substantially different classes of zymogen conformations appear to exist. In trypsinogen, the major conformational differences from trypsin are confined to the activation domain. This observation also holds for other members of the family. However, the zymogen FVII structure infers unexpected conformational differences. One is the large reorganization in the TF binding region resulting from the β strand reregistration. We set out to investigate whether the FVII zymogen structure represents the zymogen-like (latent) conformation of FVIIa, and whether the β strand hypothesis could be exploited to enhance the intrinsic activity of FVIIa. We constructed two types of FVIIa analogues designed to prevent β strand movement so that they thereby would be locked in the enzymatically active conformation. First, the first Leu-X-Val motif was modified by the introduction of Met in the third position (299). Second, a new disulfide bridge was introduced between Cys-159 and an introduced Cys at position 299 (C164V/V299C-FVIIa). If successfully locked in an active conformation (similar to that when bound to TF), the mutants would be expected to process the chromogenic substrate used approximately 50 times more efficiently than in wild-type FVIIa. The absence of an increased efficiency for any of the FVIIa variants, supported by the similar TF binding and cofactor-induced stimulation and unrestricted freedom of the N-terminus of the protease

domain, suggests that the conformational changes pertinent for the conversion of latent FVIIa to an active enzyme are confined to the activation domain.

EXPERIMENTAL PROCEDURES

Reagents and Standard Methods. Wild-type FVIIa and soluble TF (sTF) were obtained as described previously (*24*, *25*), and the concentrations of FVII(a) and sTF were determined by an ELISA and absorbance measurement at 280 nm, respectively (*26*). Factor X (FX) was from Enzyme Research Laboratories (South Bend, IN). D-Phe-Phe-Arg-chloromethyl ketone was from Calbiochem (La Jolla, CA). The chromogenic substrates S-2288 and S-2765 from Chromogenix (Mölndal, Sweden), and potassium cyanate was from Merck. SDS−polyacrylamide gel electrophoresis was carried out on a 4−12% NuPAGE Novex Bis-Tris gel (Invitrogen, Carlsbad, CA).

Mutagenesis and FVIIa Mutant Production. Wild-type FVII expression plasmid pLN174 (*27*) was the template for site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). The following primers (only sense primers shown) were used to introduce the mutations, with the base substitutions in italics and the affected codons underlined: V299M, GCCCTGGAGCTCATG*ATG*CTCAACGTGCCCCGG; L280I, CGTGCCTTCTCA*ATC*GTCAGCGGCTGGGG; C164V, CCCAAAGGGGAG*GTA*CCATGGCAGGTCC; and V299C, GCCCTGGAGCTCATG*TGC*CTCAACGTGCCC. Plasmids were prepared using the plasmid midi kit and QIA filter (Qiagen, Valencia, CA). The entire coding sequences were verified by sequencing to confirm the identities of the mutants. Baby hamster kidney cell transfection and selection and mutant expression, purification, and autoactivation were carried out as described previously (*12*, *26*).

Mass Spectrometry. Mass spectrometric peptide mapping of C164V/V299C-FVIIa was performed as follows. Wild-type and mutant FVIIa (1 μg/μL in 50 mM NH_4HCO_3) were digested in parallel with trypsin (2%, w/w) for 2 h at 37 °C, and 1 μL of the digest was applied to a MALDI target using α-cyanocinnamic acid as a matrix. A Bruker Ultraflex MALDI-TOF/TOF instrument was used to record the mass spectra. The instrument was operated in standard reflectron mode. The resulting spectra were externally calibrated using a mixture of known standard peptides. All masses are monoisotopic.

FVIIa Activity Assays. All assays were performed in 50 mM Hepes (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 1 mg/mL bovine serum albumin. The amidolytic activity of free enzyme was measured using 100 nM mutant or wild-type FVIIa, whereas the amidolytic activity when bound to sTF was measured using 10 nM FVIIa and 100 nM sTF. The substrate was S-2288 at concentrations ranging from 0.1 to 10 mM. The FX activation catalyzed by wild-type or mutant FVIIa was measured by incubating 10 nM FVIIa with 0.8 μM FX for 15 min. The reaction was terminated by the addition of assay buffer containing 20 mM EDTA, followed by the addition of S-2765 (final concentration of 0.5 mM) for quantification of FXa. The effect of potassium cyanate on the activity of mutant and wild-type FVIIa was studied by incubation of 1 μM enzyme with 0.2 M cyanate in assay buffer without albumin followed by measurement of the residual amidolytic activity as described previously (*12*). All activity measurements were carried out at 405 nm using a SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Surface Plasmon Resonance (SPR) Measurements. Mutant and wild-type FVIIa or FFR-FVIIa, in 50 mM Hepes (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$, and 0.02% Tween 80, were injected for 7 min at a flow rate of 5 μL/min and a concentration of 30 or 60 nM over a CM5 sensor chip coated with 650 resonance units of amine-coupled sTF. The subsequent dissociation phase lasted for 10 min. The instrumentation, regeneration between runs, and data evaluation were as described previously (*28*, *29*).

Structural Analyses and Molecular Modeling. Molecular analyses (and figure preparations) were performed within the frameworks of the molecular modeling package Quanta 2000 and the molecular mechanics force field CHARMm 27 (Accelrys Inc., San Diego, CA). The models of the introduced mutations have been created by mutating the side chains using the "mutation facility" within the protein modeling module in Quanta 2000. The conformations of the side chains were then adjusted using a main chain conformation-dependent rotamer library (based on high-resolution X-ray structures), followed by energy minimization [250 steps of conjugated gradient (CONJ) minimization in CHARMm].

RESULTS

Isolation of FVIIa Variants and Verification of C164V/V299C-FVIIa. The variants V299M-FVIIa, L280I/V299M-FVIIa, and C164V/V299C-FVIIa were generated by site-directed mutagenesis and stably expressed in baby hamster kidney cells. After purification from the cell culture supernatant and autoactivation, the variants were apparently homogeneous and indistinguishable from wild-type FVIIa as judged by SDS−PAGE (not shown). Mass spectrometry was employed to confirm that the mutations in C164V/V299C-FVIIa had been introduced and yielded the intended, new disulfide bond between Cys-159 and Cys-299. Figure 2A shows the MALDI-TOF spectrum of trypsin-digested FVIIa. The presence of the native disulfide link between the peptides containing residues 158−161 and 162−197 (theoretical mass of 4296.2 amu) is verified, as is the presence of peptide 291−304 (theoretical mass of 1482.8 amu). The corresponding spectrum of trypsin-digested C164V/V299C-FVIIa is shown in Figure 2B. If peptide 291−304, after introduction of the V299C mutation, was not disulfide-linked, a mass of 1486.8 amu would be detected. Alternatively, if the introduced Cys residue at position 299 was involved in a disulfide with glutathione, a mass of 1738.1 amu would be detected. Neither of these peaks is found; instead, we find a mass corresponding to peptide 291−304 disulfide-linked to peptide 158−161 (theoretical mass of 1930.0 amu). In addition, the signals corresponding to 1482.8 and 4296.2 amu are absent. This shows that the new disulfide indeed has been formed and that it is formed in all the C164V/V299C-FVIIa molecules.

Enzymatic Activity of FVIIa Variants. The amidolytic activity was measured to investigate the direct influence of the mutations on the active site functionality, whereas the proteolytic activity (activation of FX) also probes macro-

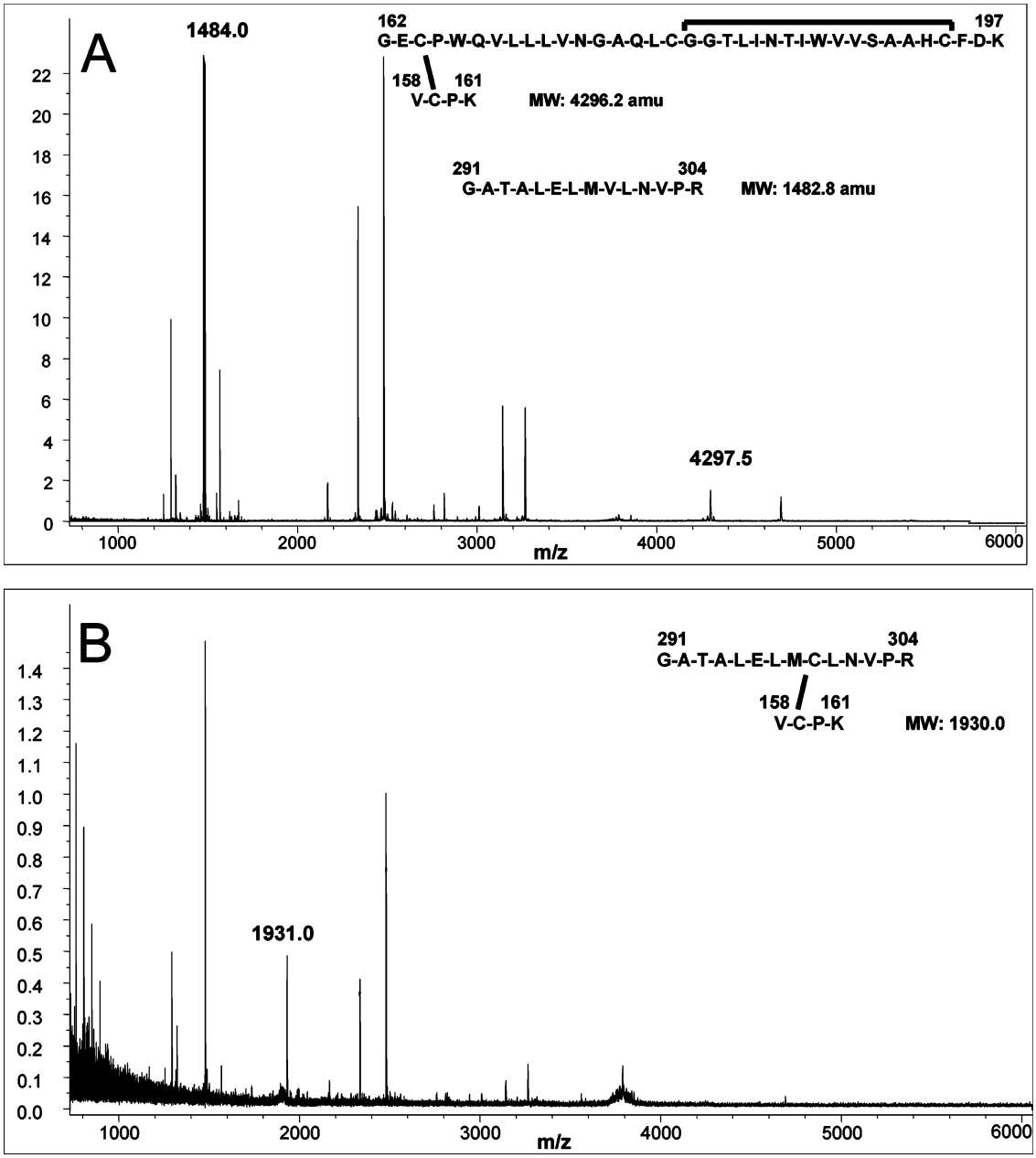

FIGURE 2: MALDI-TOF spectrum of trypsin-digested wild-type FVIIa (A) and trypsin-digested C164V/V299C-FVIIa (B). In panel A, the signals of disulfide-linked peptides 158−161 and 162−197 (theoretical mass of 4296.2 amu) and of free peptide 291−304 (theoretical mass of 1482.8 amu) are marked. In panel B, the presence of the signal of the new disulfide link connecting peptides 158−161 and 291−304 (theoretical mass of 1930.0 amu) is indicated. Notice the absence of the signals corresponding to 1482.8 and 4296.2 amu as well as the absence of signals corresponding to 1486.8 and 1738.1 amu (see the text). The dominant peak at 1477.8 amu corresponds to tryptic peptide 278−290 (theoretical mass of 1476.8 amu) and is as expected present in both panels.

molecular substrate exosite alterations. The activities of mutant and wild-type FVIIa in the absence of TF are shown in Table 1. When the parameters of the amidolytic activity were investigated, there was no significant difference between FVIIa and V299M-FVIIa in terms of k_{cat} or K_m values. The additional substitution of Ile for Leu in position 280 if anything lowered the K_m value slightly, and the double mutant L280I/V299M-FVIIa had normal kinetic parameters. If real, the slightly decreased efficiency of V299M-FVIIa may be ascribed to an unfavorable interaction of the introduced Met side chain with the surrounding protein, particularly the neighboring disulfide bridge (Cys-159−Cys-164), resulting in a slight misalignment of N-terminal insertion. The introduction of the new disulfide bridge in C164V/V299C-FVIIa decreased considerably the amidolytic activity (~2.5-fold), exclusively due to a decreased k_{cat} value.

Table 1: Chromogenic Substrate Hydrolysis and FX Activation by Free FVIIa Variants

enzyme	K_m (mM)	v_{max} (mOD/min)	v_{max}/K_m (mOD mM^{-1} min^{-1})	FX activation[a]
FVIIa	8.0 ± 0.6	69 ± 3	8.6 ± 1.0	1.00
V299M-FVIIa	9.8 ± 0.6	60 ± 2	6.1 ± 0.6	1.05 ± 0.04
L280I/V299M-FVIIa	6.6 ± 0.5	68 ± 3	10.3 ± 1.2	1.07 ± 0.02
C164V/V299C-FVIIa	9.2 ± 0.7	33 ± 1	3.6 ± 0.4	1.15 ± 0.04

[a] Rate of activation compared with that of wild-type FVIIa which is given an arbitrary value of 1. The FX concentration was 0.8 μM. Values are means ± the standard error of the mean ($n = 2$).

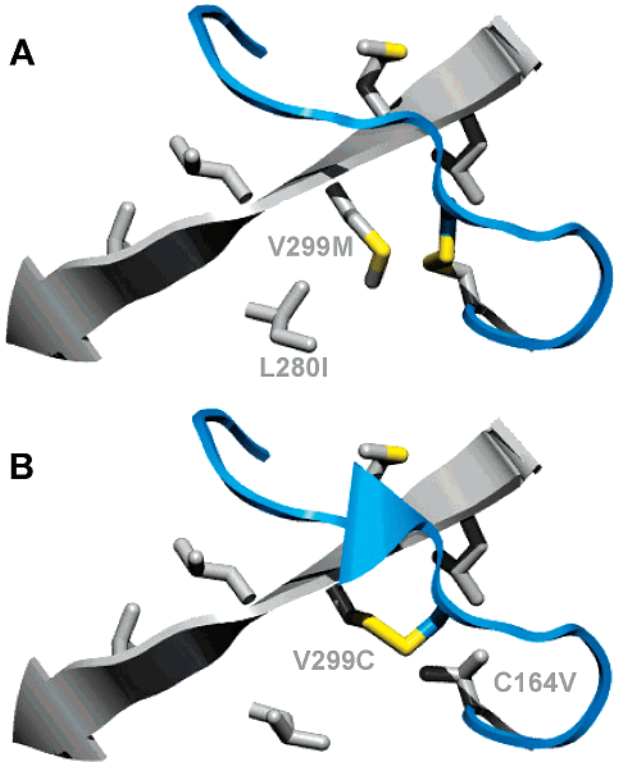

FIGURE 3: Models of mutants L280I/V299M-FVIIa (A) and C164V/V299C-FVIIa (B). β strand B2 in the active FVIIa conformation, residues 296−304, is colored gray, and N-terminal residues 153−165 are colored blue. The model in panel A has been generated by mutating the side chains *in silico* and adjusting the conformations by consulting rotamer libraries, followed by energy minimization to relieve steric clashes. In panel B, mutations C164V and V299C were introduced *in silico* to mimic the situation in bovine trypsin or human kallikrein. Formation of the new disulfide bridge, followed by application of rotamer libraries and energy minimization, resulted in the depicted model. The conformation of the disulfide is almost identical to those observed in the X-ray structures of bovine trypsin and human kallikrein.

This reduction in activity is most likely due to a minor rearrangement of the N-terminus which weakens the insertion. The mutant constructs are illustrated in Figure 3, and the proximity of the mutated positions to the N-terminus is clear. Interestingly, the proteolytic activity of all variants, including C164V/V299C-FVIIa which displayed a lower amidolytic activity, was normal or possibly marginally increased compared to that of FVIIa (Table 1). The activity measurements suggest that the modifications in V299M-, L280I/V299M-, and C164V/V299C-FVIIa are tolerated and leave FVIIa in a functionally native state, apart from a slightly lower amidolytic activity in one case. We also investigated whether the mutations affect the exposure of the N-terminus of the protease domain. The susceptibility to chemical modification (carbamylation) by potassium cyanate, measured as a reduction in amidolytic activity, was similar in L280I/V299M-FVIIa, C164V/V299C-FVIIa, and wild-type FVIIa. All three molecular species retained between 33 and 40% of the activity following incubation for 1 h. This indicates that the mutations do not alter the propensity of the N-terminal amino group to form a salt bridge with the side chain of Asp-343.

FVIIa·TF Interaction and Allostery. The functional state of the variants appeared to be unaltered as judged from the enzymatic activity measurements and as inferred from the carbamylation data. To further corroborate that the mutants were in a conformation, or functional state, similar to that of wild-type FVIIa, we studied TF binding, the ability of sTF to stimulate the amidolytic activity, and the effect of active site inhibitor incorporation on the affinity for sTF. After complex formation with sTF, all three FVIIa variants achieved an activity similar to that of wild-type FVIIa (Table 2). This suggests that sTF binding is able to correct for the slight functional impairment of free C164V/V299C-FVIIa. As measured by SPR, L280I/V299M-FVIIa and C164V/V299C-FVIIa bound sTF with K_d values of 2.6 and 4.2 nM, respectively. These values are similar to that obtained with wild-type FVIIa (3.4 nM), indicating that the TF binding region is indistinguishable in the three forms of FVIIa and neither perturbed nor improved by the mutations. Incorporation of active site inhibitor FFR-ck is known to increase the affinity of FVIIa for sTF, lowering K_d to <1 nM (*21*, *22*). We here find that the interaction between FFR-FVIIa and sTF is characterized by a K_d of 0.5 nM, whereas L280I/V299M-FFR-FVIIa and C164V/V299C-FFR-FVIIa bind sTF with K_d values of 0.8 and 0.9 nM, respectively. The indistinguishable activities in complex with sTF and similar affinities for sTF after incorporation of FFR-ck strongly suggest that the same allostery occurs in all three species of FVIIa.

Table 2: Chromogenic Substrate Hydrolysis by FVIIa Variants in the Presence of sTF

enzyme	K_m (mM)	v_{max} (mOD/min)[a]	v_{max}/K_m (mOD mM^{-1} min^{-1})
FVIIa	1.5 ± 0.1	41 ± 1	27.3 ± 2.5
V299M-FVIIa	2.3 ± 0.2	50 ± 2	21.7 ± 2.8
L280I/V299M-FVIIa	2.0 ± 0.2	47 ± 2	23.5 ± 3.3
C164V/V299C-FVIIa	2.2 ± 0.2	39 ± 2	17.7 ± 2.5

[a] Values are means ± the standard error of the mean (n = 2).

DISCUSSION

Enzymatically active FVIIa adopts a conformation which is virtually identical to that of trypsin (*18*). Hence, the structure of the activation domain and the active site region, as well as the overall topology, are conserved as judged from crystal structures. In contrast, large differences are observed between the structures of zymogen FVII (*23*) and trypsinogen (*4*). The activation domain of FVII is quite different from that of trypsinogen, and in particular, large differences are observed in the TF binding region resulting from the reregistration of β strand B2. On the basis of the structure of FVII, it was suggested that binding of TF to FVIIa promotes a three-residue shift of β strand B2 for attainment of the enzymatically active conformation (*23*). The Leu-X-Val repeats were hypothesized to favor the reregistration (Figure 4). One remaining question is whether the structure of FVII resembles or corresponds to the latent structure of FVIIa.

If β strand reregistration is an integral part of the transition between the zymogen-like and enzymatically active conformations, locking β strand B2 in the "active position" should result in FVIIa activity enhancement. To investigate this possibility, appropriate mutations aimed at locking the protease domain in the conformation seen in active site-inhibited FVIIa complexed with TF were introduced (*18*).

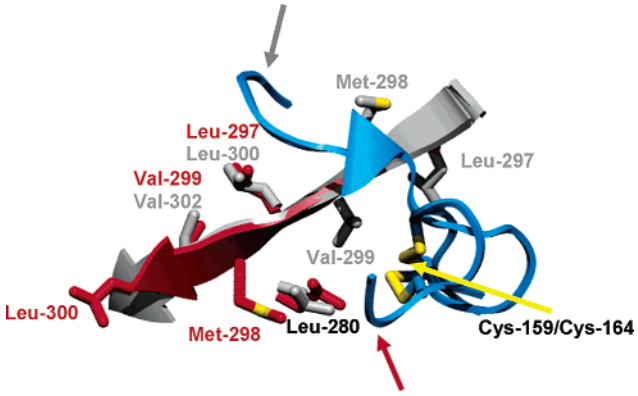

FIGURE 4: Reregistration of β strand B2. Superposition of residues 296−304 in gray comprising β strand B2 of the active FVIIa conformation and residues 294−300 in red comprising β strand B2 of the zymogen FVII conformation. Shown in blue are parts of the N-termini: residues 153 (gray arrow) through 165 of active FVIIa and residues 157 (red arrow) through 165 of zymogen FVII. The position of the disulfide bridge between Cys-159 and Cys-164 is virtually identical in the two structures (yellow arrow). As a consequence of the reregistration, the position of the Cα atom of Met-298 in the active FVIIa conformation (gray) has shifted 11.5 Å relative to the position in the zymogen conformation (red). The same shift is observed for the leucine and valine residues in the Leu-Met-Val-Leu-Asn-Val (residues 297−302) motifs.

The effects of the mutations on the intrinsic properties of FVIIa were assessed in activity assays, TF binding, and N-terminus accessibility measurements capable of capturing the impact on the active site, the macromolecular substrate exosite, and the TF-interactive region. The influence of the mutations on the allosteric effects of TF and active site occupancy were also studied in terms of cofactor-mediated activity enhancement and an inhibitor-induced TF affinity increase.

The first attempt to stabilize the enzymatically active FVIIa conformation by mutagenesis was based on the idea that disruption of the first of two Leu-X-Val repeats would prevent the transition from an active to a zymogen-like conformation. To accomplish this, valine in position 299 was replaced with methionine. Methionine would presumably experience a steric clash if the β strand shifted by three residues, in particular to the side chains constituting the cavity surrounding Val-302 in the active form of FVIIa (*18*). To test this hypothesis, the V299M mutation was introduced *in silico* into the zymogen structure (see Experimental Procedures). Because of the bulkiness of the methionine, several neighboring main chain and side chain atoms moved relative to their positions in the native zymogen structure, e.g., main chain atoms in the loop of residues 331−333 (1.4 Å) and side chain atoms of Leu-297 and Tyr-377 (1.5 Å). The hydrogen bonds stabilizing the β sheet were however intact. The *in silico* V299M mutation led to much smaller structural changes in the enzymatically active conformation; i.e., only close contacts with Leu-280 were observed. Hence, the observation that V299M disturbs the zymogen structure but only marginally changes the active conformation points to the conclusion that the V299M mutation favors the latter conformation. In this context, it is worth mentioning that a comparison of the two native structures reveals that the solvent exposure of nonpolar side chains is much larger in the zymogen structure than in the active structure. Likewise, polar side chains tend to be less solvent exposed in the zymogen structure than in the enzymatically competent structure (*23*). Thus, the active conformation seems to be favored which points to the suggestion that the dramatic changes in the sTF region of the zymogen structure compared to the active structure are promoted by the presence of the allosteric inhibitor. The amidolytic activity of free and TF-bound V299M-FVIIa, as well as its intrinsic proteolytic activity, was found to be similar to that of wild-type FVIIa. It is conceivable that the (only) normal activity was due to a clash between the introduced Met in position 299, when in the "active position", and Leu-280 and/or the disulfide bridge between Cys-159 and Cys-164, resulting in a slight misalignment of the insertion of the N-terminus into the activation pocket (Figure 3). To rule out this possibility, the L280I mutation was also introduced. The double mutant L280I/V299M-FVIIa resembles, for instance, FXa in these two positions. However, L280I/V299M-FVIIa was not significantly different from FVIIa and V299M-FVIIa in terms of proteolytic and amidolytic activity. Moreover, the rate of N-terminal carbamylation is indistinguishable from that of FVIIa. Our results suggest that the free forms of wild-type FVIIa, V299M-FVIIa, and L280I/V299M-FVIIa are in the same conformational, and consequently activity, state and that TF exerts the same allosteric stimulatory effect. In addition, the finding that TF has the same affinity for FVIIa and L280I/V299M-FVIIa, both before and after chloromethyl ketone inhibition, strongly suggests that the TF binding region is unaltered in the mutant and does not lend support to differences in relative β strand positions. In other words, the disruption of the first Leu-X-Val repeat does not appear to affect the TF binding region. This can be explained in two ways. Either the β strand reregistration occurs despite the V299M mutation, or the β strand shift is not an integral part of the transition between the active and zymogen-like conformations of FVIIa. It is noteworthy that a comparison of available FVII sequences from various species shows that only human FVII contains two consecutive Leu-X-Val repeats. In the third position of the first repeat, one occasionally finds serine or alanine, while the first position of the second repeat sometimes is occupied by isoleucine or valine. Even though this observation can support both explanations, it is likely that FVIIa in all species employs the same activation mechanism, suggesting that the three-residue shift does not play a role in the transition to and from a more active conformation.

To corroborate our findings regarding the relevance of the β strand shift, we decided to construct a FVIIa variant with a dramatically restricted neighborhood around the β strand. Cys-159 participates in two different disulfide bridge arrangements in the trypsin family of serine proteases. FVIIa (and FXa) contains the Cys-159−Cys-164 disulfide, while the others (e.g., trypsin) contain a bridge between Cys-159 and Cys-299. On the basis of the notion that the different patterns do exist, as well as on the basis of modeling of the latter disulfide pattern into the FVIIa structure (Figure 3), we constructed C164V/V299C-FVIIa. Importantly, the position of the Cys-159−Cys-164 disulfide is quite similar in the zymogen FVII structure and in the enzymatically active structure of FVIIa (Figure 4). Hence, the arrangement of hydrophobic side chains (in particular, Pro-165 and Trp-166) surrounding the Cys-159−Cys-164 disulfide is conserved in the two structures. The buried side chains of Pro-165 and

Trp-166 are stabilized via hydrophobic interactions to structurally conserved light chain residues Ile-138 and Pro-139 and to Trp-356 in the protease domain. The aliphatic part of the Glu-163 side chain in the enzymatically competent structure interacts with light chain residues Ile-140 and Leu-141. Because of coiling of the N-terminus in the zymogen structure, this interaction is taken up by Pro-160 which stabilizes the zymogen conformation. Again, modeling experiments were performed to elucidate the impact of the C164V and V299C mutations on the zymogen structure. The altered disulfide pattern has consequences for key interactions seen in the zymogen FVII structure. First, the Leu-X-Val repeat has been more profoundly affected than in the L280I/V299M mutant because in the alleged latent conformation the disulfide bridge and parts of the N-terminal tail have to fit the cavity occupied by Val-302 in the active conformation. Second, the hydrogen bonds involving the carboxylate of Glu-296, which were suggested to be the major reason for the stabilization of the zymogen structure (*23*), are no longer feasible because the donor atoms (the main chain amide nitrogens of Cys-159 and Pro-160) have been translated 10 Å due to the β strand shift. Similarly, the remaining part of Pro-160 must translate at least 10 Å due to the β strand shift, thereby losing the stabilizing interaction with Ile-140 and Leu-141. However, the most dramatic effect of the new disulfide is that buried residues Pro-165 and Trp-166 also must translate to maintain the β strand shift. The notion that the structural arrangement of these buried residues is conserved in the two structures makes us suggest that the β strand shift is highly unlikely to occur in C164V/V299C-FVIIa. The formation of the new disulfide was verified by means of mass spectroscopy, and the intrinsic amidolytic activity of C164V/V299C-FVIIa was approximately half of that of wild-type FVIIa, whereas the proteolytic activity was normal. Remodeling of a macromolecular substrate exosite has perhaps compensated for a less mature active site. In addition, sTF largely restored the amidolytic activity to normal (Tables 1 and 2). The observed reduction in amidolytic activity is most likely due to a minor rearrangement of the N-terminus which affects its insertion. However, this is barely supported by the carbamylation data. The fact that sTF binds with unaltered affinity to the mutant, also after incorporation of an active site inhibitor, and restores the activity points to the conclusion that the TF binding region is unperturbed. In a recent study (*30*), the binding of a panel of 12 sTF mutants to zymogen FVII and FVIIa was evaluated. Albeit altered, the affinities for FVII and FVIIa were comparable, and it was concluded that zymogen FVII and FVIIa interact with sTF in a nearly identical fashion. These data can be interpreted in two ways; either the zymogen FVII can readily adopt a FVIIa-like conformation (to bind sTF), or the structural difference between zymogen FVII and active FVIIa is subtle.

The body of data obtained with C164V/V299C-FVIIa points to the same conclusion as for L280I/V299M-FVIIa, namely, that the equilibrium between the zymogen-like and active conformations of the mutant is indistinguishable from that of FVIIa. In particular, the mutants showed no signs of increased intrinsic activity as anticipated if the β strand positioning is important for the activity. These experimental findings, in combination with the structural constraints introduced by the mutations preventing β strand reregistration, make us conclude that the dramatic structural differences in terms of β strand positioning between FVII and FVIIa (TF-bound and/or active site-inhibited) are not involved (or do not suffice) in the mechanism that governs the activity state of FVIIa. In contrast, our data raise the possibility that the structural differences between the latent (zymogen-like) and active structures of FVIIa resemble those between trypsinogen and trypsin. Hence, we expect the structural differences between the two conformations of FVIIa to be confined to the activation domain, including the N-terminal insertion pocket. We hypothesize that the allosteric effects of TF only materialize in subtle conformational changes. Small structural changes with a dramatic impact on enzymatic activity have been elucidated for thrombin (*31*), where concerted movements of the Cys-168c−Cys-182c (c for chymotrypsinogen numbering) disulfide bridge and the neighboring side chains of Phe-227c, Trp-215c, and Trp-60Dc constitute an allosteric switch which opens and closes the substrate binding cleft. The corresponding region of FVIIa is in the vicinity of the TF binding region, and an allosteric mechanism similar to that observed for thrombin can be responsible for the transmission of the signal from TF to the active site region of FVIIa.

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