The Origins of Enhanced Activity in Factor VIIa Analogs and the Interplay between Key Allosteric Sites Revealed by Hydrogen Exchange Mass Spectrometry

Kasper D. Rand, Mette D. Andersen, Ole H. Olsen, Thomas J. D. Jørgensen, Henrik Østergaard, Ole N. Jensen, Henning R. Stennicke, and Egon Persson

From the 1Department of Haemostasis Biochemistry, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark and the 2Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

Factor VIIa (FVIIa) circulates in the blood in a zymogen-like state. Only upon association with membrane-bound tissue factor (TF) at the site of vascular injury does FVIIa become active and able to initiate blood coagulation. Here we used hydrogen exchange monitored by mass spectrometry to investigate the conformational effects of site-directed mutagenesis at key positions in FVIIa and the origins of enhanced intrinsic activity of FVIIa analogs. The differences in hydrogen exchange of two highly active variants, FVIIaDVQ and FVIIaVEAY, imply that enhanced catalytic efficiency was attained by two different mechanisms. Regions protected from exchange in FVIIaDVQ include the N-terminal tail and the activation pocket, which is a subset of the regions of FVIIa protected from exchange upon TF binding. FVIIaDVQ appeared to adopt an intermediate conformation between the free (zymogen-like) and TF-bound (active) form of FVIIa and to attain enhanced activity by partial mimicry of TF-induced activation. In contrast, exchange-protected regions in FVIIaVEAY were confined to the vicinity of the active site of FVIIa. Thus, the changes in FVIIaVEAY appeared to optimize the active site region rather than imitate the TF-induced effect. Hydrogen exchange analysis of the FVIIaM306D variant, which was unresponsive to stimulation by TF, correlated widespread reductions in exchange to the single mutation in the TF-binding region. These results reveal the delicate interplay between key allosteric sites necessary to achieve the transition of FVIIa into the active form.

Factor VIIa (FVIIa) contains a trypsin-like protease domain connected via a disulfide bond to a light chain composed of an N-terminal domain rich in γ-carboxyglutamic acid residues (Gla domain) and two epidermal growth factor-like domains (EGF1 and EGF2) (1). Upon vascular injury, FVIIa circulating in the blood binds to tissue factor (TF) exposed on extravascular cells and initiates blood coagulation. TF acts as an allosteric regulator and dramatically enhances the activity of FVIIa (2). Similar to most trypsin-like proteases, single-chain zymogen FVII is proteolytically cleaved at a peptide bond adjacent to the protease domain to become the two-chain FVIIa molecule. In trypsin, the new N terminus (residue 16) generated upon endopeptidase of trypsinogen spontaneously inserts into a cavity termed the activation pocket and forms a salt bridge with residue 194. This key step leads to the formation of a correctly shaped S1 pocket of the active site and full catalytic activity. Crystal structures of the trypsin/trypsinogen pair indicate that three loops (referred to as the activation loops) spanning the surface between the activation pocket and the active site become ordered upon activation (3, 4). FVIIa, however, retains zymogen-like properties after cleavage and exhibits very low enzymatic activity. There is biochemical evidence that the new N-terminal Ile-153(16) of FVIIa (the chymotrypsin numbering is denoted in superscript with parentheses) fails to insert into the S1 pocket of FVIIa and that TF binding facilitates N-terminal insertion and transition to the catalytically competent form of FVIIa (5, 6). Crystal structures of FVIIa in the presence of TF and active site inhibitors provide detailed information on the active form of FVIIa (Fig. 1) (7–9). Recently, insights into zymogen FVII and the zymogen-like form of FVIIa were obtained by probing the solution structures of these two activation states, and that of TF-bound FVIIa, by hydrogen exchange monitored by mass spectrometry (HX-MS) (10). The conformations of zymogen FVII and FVIIa were highly similar and confirmed the zymogenicity of FVIIa, whereas TF binding induced widespread conformational changes in multiple regions of the protease domain, e.g. the N terminus, the activation pocket, and the activation loops (10).

Pharmacological doses of recombinant FVIIa can ameliorate the hemostatic defect of patients with hemophilia A or B and inhibitory antibodies. Promising results have also been obtained with FVIIa in non-hemophilic patients after traumatic injury (11, 12). High doses of FVIIa are necessary for treating bleeding episodes in these patients, because FVIIa presumably exerts at least part of its function without TF (13, 14). New
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FVIIa variants with enhanced intrinsic activity could therefore potentially be beneficial by offering improved hemostasis. We have discovered several such variants through site-directed mutagenesis in the protease domain of FVIIa and have selected four FVIIa analogs with unique properties for the present study. These are FVIIa-M306D, which is unresponsive to activation by TF (15), FVIIa-M306D, which has optimized activity especially toward small peptidyl substrates (16), and FVIIa-M306D and FVIIa-M306D, with dramatically improved activity toward the natural macromolecular substrate factor X (FX) (17–19).

FVIIa-M306D contains four mutations in the protease domain, L305(V163, V), S314(E170, E), K337(A188, A), and F374(Y225, Y) and processes small peptidyl substrates very efficiently with a higher apparent catalytic rate and a reduced Km compared with FVIIa (16) (Table 1). Similarly, the intrinsic activity toward FX is increased 10-fold. Similar to FVIIa-M306D, FVIIa-M306D possesses increased amidolytic activity toward small peptidyl substrates (predominantly a higher apparent kcat), but most important, it displays a 40–50-fold increased rate of FX activation (17). The three mutations in FVIIa-M306D are situated in the N-terminal tail of the heavy chain (V158(D21)D) and near the activation pocket (E296(Y154)V and M298(Y156)Q) and mimic a sequence motif from thrombin and factor IXa. The related FVIIa-M306D variant has properties similar to those of FVIIa-M306D but with a further increased activity due to the additional K337(A188)A mutation (17). In contrast to these highly active variants, FVIIa-M306D has an intrinsic activity comparable with that of FVIIa. The variant has a single M306(V163)D mutation in the TF-binding region of the protease domain. Met-306(A164) is the most locally influential residue both in terms of TF binding energy and mediating the allosteric effect of TF (15, 20). In addition, mutation of Met-306(A164) abolishes or diminishes the affinity increase toward TF that is induced by incorporation of an active site inhibitor into FVIIa (15, 21). The salient property of this variant is that, although FVIIa-M306D and FVIIa bind TF with comparable affinity, the activity is virtually unaffected by TF binding demonstrating an abrogated allosteric response to TF (15).

Structural information relating to the intrinsic properties of the individual variants is lacking as it would require the crystallization of free, uninhibited FVIIa, which so far has eluded all attempts. Detailed information about the conformational properties and dynamics of proteins in solution is contained in their HX behavior, and HX rates of native proteins are a sensitive measure of local conformational stability (22, 23). HX-MS has become a widely applicable technique, which affords rapid monitoring of protein structural transitions in solution relating to folding, function, and regulation (24). In this study, we applied HX-MS analyses on a set of FVIIa variants that spanned the entire spectrum of properties obtained by site-directed mutagenesis. The HX profiles of the FVIIa variants were used to provide a structural rationale for the origins of their enhanced intrinsic activity. Moreover, combining the HX analyses allowed us to identify key allosteric sites in the protease domain, which are linked by an intricate network of dynamic cross-talk.

**EXPERIMENTAL PROCEDURES**

**HX Reactions and MS Analysis**—FVIIa-DVQA, FVIIa-DVQA, FVIIa-M306D, and FVIIa-M306D were obtained as described (15–17). Sample preparations of FVIIa variants and soluble human TF-(1–219) (sTF) were done as described previously (10). Amide 1H-1H HX was performed exactly as described earlier (10) and initiated by a 12-fold dilution of a protiated protein stock solution in the presence or absence of cofactor into the corresponding deuteration buffer (i.e., 20 mM bis-Tris, pH 6.0 (uncorrected value), 10 mM CaCl2, 99% D2O). HX reactions were performed at pH 6.0 to better resolve the fast exchanging amides. Non-deuterated controls were prepared by dilution into an identical protiated buffer. All HX reactions were carried out at 25 °C and contained 4.3 μM FVIIa variant in the absence or presence of 21 μM sTF. At appropriate time intervals, aliquots of the HX reaction were quenched by the addition of an equal volume of ice-cold quenching buffer (1.25 M Tris(2-carboxyethyl)phosphine hydrochloride, adjusted to pH 2.15 using NaOH) resulting in a final pH of 2.3 (uncorrected value). Quenched samples were immediately frozen in liquid N2 and stored at −80 °C. The apparatus used for rapid desalting and subsequent mass analysis was configured as described (10). Positive ion-electrospray ionization mass spectra of eluted peptides were acquired on a LCT mass spectrometer (Waters Corp.).

**Data Analysis**—Peptic peptides were identified in separate experiments using standard collision-induced dissociation tandem mass spectrometry (CID MS/MS) methods on a Q-TOF 1 instrument (Waters). Average masses of peptide isotopic envelopes were determined from lock mass-corrected centroided data (processed using MassLynx software, Waters) using an Excel spreadsheet. Complete deuteration of control samples was achieved by incubation for 70 h at 37 °C in the presence of 6 M guanidinium chloride (deuterated). Average back exchange (i.e., deuterium loss) was measured as 14% for the analyzed peptides. However, no corrections were made for this deuterium loss because only the relative levels of deuterium incorporation of all samples were compared. All HX experiments were repeated at least twice. Replicate samples of FVIIa after a 100-
or 300-s exchange indicated a standard deviation of 0.2 deuterium \( (n = 3) \) in the determination of peptide deuterium contents. Significant changes in deuterium incorporation were therefore defined as a difference larger than the double standard deviation, i.e. > 0.4 deuteriums. Protein structures were visualized using the software PyMOL (DeLano Scientific).

Deglycosylation of FVIIa and FVIIa\(_{M306D}\)–PNGase F–catalyzed cleavage of the N-glycosidic linkage to Asn-322\(^{175}\) in the protease domain was monitored by HPLC for FVIIa\(_{M306D}\) in the free form and in complex with sTF as described previously for FVIIa (25). Deglycosylation of FVIIa\(_{M306D}\) was carried out at 30 °C in a total volume of 100 \( \mu \)l of buffer (50 mM HEPES, 100 mM NaCl, 10 mM CaCl\(_2\), 0.01% Tween 80, pH 7.0) containing 3 mM tris(2-carboxyethyl)phosphine and 10% formic acid was quenched by 8-fold dilution into a solution consisting of 70 mM tris(2-carboxyethyl)phosphine and 10% formic acid.

| Wild type       | Amidolytic activity | PABA inhibition \( K_i \) | Activity loss per 10 min carbamylation | Rate of deglycosylation of Asn-322\(^{175}\) |
|-----------------|---------------------|---------------------------|---------------------------------------|---------------------------------------------|
| relative \( k_{cat} \) \( a \) | relative \( k_{cat} \) \( a \) | %                         | %                                     | %                                         |
| Wild type       | 1 (10)              | 2.2 (0.09)                | 15–19 (5–8)                           | 45.7 (0.46)                                |
| FVIIa\(_{DVQ}\) \( b \) | 4 (10)              | 0.23 (0.05)               | 2–3 (ND)                              | ND                                         |
| FVIIa\(_{DVQA}\) | 4 (12)              | 0.18 (0.04)               | 15–17 (6–8)                           | ND                                         |
| FVIIa\(_{M306D}\) | 0.8 (2)             | 2.4 (1.7)                 | 15–18 (15–17)                         | 19.5 (11.5)                                |

\( a \) \( k_{cat} \) values were measured using the substrate S-2288 and are relative to that of free FVIIa (which is given the arbitrary value of 1). The relative \( k_{cat} \) values given are apparent and could have contributions from both \( k_{cat} \) and \( E_0 \) (effective enzyme concentration).

\( b \) Except for a 2-fold increase in amidolytic activity, the biochemical properties of FVIIa\(_{DVQA}\) were highly similar to those of FVIIa\(_{DVQ}\) (17).

### RESULTS

**HX Profiling of Functionally Distinct Variants of FVIIa**—The variants FVIIa\(_{VEAY}\), FVIIa\(_{DVQ}\), and FVIIa\(_{M306D}\) were previously shown to be bestowed with distinct biochemical properties, in particular increased amidolytic and proteolytic activity and abrogated activation by TF, respectively. In addition to activity measurements, FVIIa\(_{VEAY}\) and FVIIa\(_{DVQ}\) were characterized in experiments that indirectly probed the structure of the protease domain by measuring the propensity for insertion of the N terminus (susceptibility of the amino group of Ile-153\(^{16}\) to carbamylation) or by assessing the functionality of the S1 pocket by PABA inhibition (16, 17). We subjected FVIIa\(_{M306D}\) to the same tests; an overview of the available biochemical data on the FVIIa variants is presented in Table 1.

In search of a detailed structural rationale for the unique properties of these FVIIa variants and to gain insight into the functional dynamics of the FVIIa protease domain, we subsequently probed each variant for sites of conformational changes by HX–MS analysis. HX was initiated by dilution of the protein into deuterated buffer and deuterium labeling of accessible sites in the protein was allowed to proceed for various exchange times. Localization of deuterium labels on amide groups of the protein backbone was subsequently achieved by denaturation and digestion with pepsin under acidic HX quench conditions to retain the deuterium labels. The resulting peptic peptides were separated by HPLC, and the time-resolved deuterium content of each peptide were analyzed by electrospray MS. In this manner, 24 corresponding peptides from the protease domain were identified for FVIIa\(_{VEAY}\), FVIIa\(_{DVQ}\), FVIIa\(_{DVQA}\), and FVIIa\(_{M306D}\) (Fig. 2a and supplemental Fig. 1, a and b) and used to monitor the HX for 225 of a total of 253 backbone amide hydrogens (90% of the FVIIa protease domain). Replicate HX

### Table 1

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|----------------------------------------------|
| The biochemical data listed on FVIIa, FVIIa\(_{DVQ}\), and FVIIa\(_{VEAY}\) were compiled from previous studies (16, 17, 25). Values measured in the presence of sTF are indicated in parentheses. ND, denotes values that were not determined. |

### Table 1

| FVIIa variant | Amidolytic activity | PABA inhibition \( K_i \) | Activity loss per 10 min carbamylation | Rate of deglycosylation of Asn-322\(^{175}\) |
|---------------|---------------------|---------------------------|---------------------------------------|---------------------------------------------|
|               | relative \( k_{cat} \) \( a \) | relative \( k_{cat} \) \( a \) | % | % |
| Wild type     | 1 (10)              | 2.2 (0.09)                | 15–19 (5–8)                           | 45.7 (0.46)                                |
| FVIIa\(_{DVQ}\) | 4 (10)              | 0.23 (0.05)               | 2–3 (ND)                              | ND                                         |
| FVIIa\(_{DVQA}\) | 4 (12)              | 0.18 (0.04)               | 15–17 (6–8)                           | ND                                         |
| FVIIa\(_{M306D}\) | 0.8 (2)             | 2.4 (1.7)                 | 15–18 (15–17)                         | 19.5 (11.5)                                |

\( a \) \( k_{cat} \) values were measured using the substrate S-2288 and are relative to that of free FVIIa (which is given the arbitrary value of 1). The relative \( k_{cat} \) values given are apparent and could have contributions from both \( k_{cat} \) and \( E_0 \) (effective enzyme concentration).

\( b \) Except for a 2-fold increase in amidolytic activity, the biochemical properties of FVIIa\(_{DVQA}\) were highly similar to those of FVIIa\(_{DVQ}\) (17).
measurements indicated a standard deviation of 0.2 deuteriums (n = 3) in determination of peptide deuterium contents. An example of raw HX-MS data, in this case on peptide 6 (residues 207–220(67–80)) of FVIIa VEAY, FVIIaDVQ, and FVIIa after exchange for 10 s, is shown in Fig. 2b. Each peptide reports on the HX of all of its amide hydrogens (side-chain hydrogens are not detected) except for the N-terminal amide hydrogen (26). The HX profiles of the FVIIa variants, in the presence and absence of TF, revealed significantly reduced HX in several different peptides, suggestive of conformational stabilization in distinct regions relative to FVIIa. Although the HX of the light chain of the FVIIa analogs was largely identical to that of FVIIa (data not shown),4 the protection from HX could be localized to numerous functionally important segments of the FVIIa protease domain, many of which were previously found to be exchange-protected during the allosteric response induced by TF binding (10) (Fig. 3).

**HX Behavior of the Highly Active FVIIaDVQ Variant**—The three mutations in FVIIaDVQ, situated in the N-terminal tail of the heavy chain (V158(21)D) and near the activation pocket (E296(154)V and M298(156)Q), have a profound effect on the HX of several regions of the protease domain (Fig. 3b). Locally, at the site of mutagenesis, the N-terminal tail (peptide 1: residues 153–169(16–32), Fig. 4) displayed reduced HX, indicating a stabilization of this key part of the protease domain (Fig. 1). In support of this observation, the loss of activity of FVIIaDVQ upon carbamylation occurred drastically slower compared with FVIIa, confirming an increased burial of the N terminus in this analog (Table 1). However, protection from HX was also observed in neighboring regions of the protease domain. This includes β-strand A2 (peptide 11: residues 275–287(129F-144), Fig. 4) forming part of the activation pocket for N-terminal insertion (Fig. 1). Likewise, moderately reduced HX was observed in neighboring regions of the protease domain. This includes β-strand A2 (peptide 11: residues 275–287(129F-144), Fig. 4) forming part of the activation pocket for N-terminal insertion (Fig. 1). Likewise, moderately reduced HX was observed in neighboring regions of the protease domain. This includes β-strand A2 (peptide 11: residues 275–287(129F-144), Fig. 4) forming part of the activation pocket for N-terminal insertion (Fig. 1).
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between the active site cleft and the activation pocket (Fig. 1). The Ca²⁺-binding loop (peptide 5: residues 207–220(67–80), Fig. 4), whose conformation and binding of Ca²⁺ has been correlated to increased activity of several trypsin-like proteases including FVIIa (27–30) also underwent slower exchange in FVIIaDVQ compared with FVIIa. As expected, the related FVIIaDVQA variant displayed very similar HX behavior to FVIIaDVQ. However, although FVIIaDVQ displayed only minor protection from HX in activation loop 2 (peptide 18: residues 332–356(184–207); Fig. 5), the corresponding region of FVIIaDVQA, which contains the additional K337(188)A mutation, clearly underwent substantially slower HX.

HX Behavior of the Highly Active FVIIaVEAY Variant—The four mutations in the FVIIaVEAY analog are located in three loops of the active site region: the 170 loop (S314(170B)E), activation loop 2 (K337(188)A) and activation loop 3 (F374(225)Y) and in the TF-binding site (L305(163)V). The HX profile of FVIIaVEAY showed that several segments of the protease domain were protected from HX relative to the wild-type enzyme (Fig. 3c). Moderate reductions in HX were observed in activation loop 2 (peptide 18: residues 332–356(184–207; Fig. 5), activation loop 3 (peptide 20: residues 361–377(212–228); Fig. 5), and in the 170 loop (peptide 16: residues 311–325(169–178); Fig. 5). The latter loop borders the S3 and S4 sites of the substrate cleft, and replacement of the 170 loop with the corresponding segment from trypsin has previously been shown to result in increased amidolytic activity (31). Furthermore, more pronounced reductions in HX were observed in activation loop 2 (peptide 18: residues 332–356(184–207; Fig. 5), activation loop 3 (peptide 20: residues 361–377(212–228); Fig. 5), and in the 170 loop (peptide 16: residues 311–325(169–178); Fig. 5). The latter loop borders the S3 and S4 sites of the substrate cleft, and replacement of the 170 loop with the corresponding segment from trypsin has previously been shown to result in increased amidolytic activity (31).

FIGURE 3. Structural stabilization of three variants of FVIIa as revealed by HX. Left and right panels show a schematic of the FVIIa protease domain from two different orientations (adapted from the x-ray structure of the FVIIa-TF complex (7); Protein Data Bank code 1DAN). a, structural representation of FVIIa indicating locations of peptides with reduced HX upon TF binding(10). b–d, structural representations of the FVIIaDVQ, FVIIaVEAY, and FVIIaM306D variants, respectively, indicating the location of peptides with reduced HX relative to FVIIa. Mutation sites are indicated by asterisks. Peptides with unaffected HX are shown in white, and peptides with reduced HX relative to FVIIa are shown in color according to the coloring scheme used in Fig. 2. The activation loops are numbered, and the N and C termini are indicated. Residues of the catalytic triad and the bound Ca²⁺ ion are shown in yellow. Peptides for which no HX data were obtained are shown in black.

observed in activation loop 1 (sublocalization via peptides 12 and 13, supplemental Fig. 1, a and b), activation loop 2 (peptide 18: residues 332–356(184–207). Fig. 5) and activation loop 3 (peptide 20: residues 361–377(212–228). Fig. 5), all three closely situated on one face of the protease domain spanning the region

the activation pocket. Notably, the N-terminal tail (peptide 1: residues 153–169(16–32). Fig. 4) and the neighboring Ca²⁺-binding loop (peptide 5: residues 207–220(67–80). Fig. 4) of this analog displayed HX indistinguishable from that of FVIIa. In conjunction, carbamylation experiments on FVIIaVEAY indicated
similar susceptibility of the N terminus to modification as that of the wild-type enzyme (Table 1).

**HX Behavior of the TF-unresponsive FVIIaM306D Variant**—
The FVIIaM306D variant has a single M306(164)D mutation in the TF-binding region of the protease domain. As illustrated in Fig. 3d, this has quite a dramatic effect on the HX of numerous regions of the protease domain of FVIIaM306D. Pronounced HX reductions were observed in the loops of the active site region, including activation loop 2 (peptide 18: residues 332–356(184–207); Fig. 5), activation loop 3 (peptide 20: residues 361–377(212–228); Fig. 5), and the 170 loop (peptide 16: residues 311–325(169–179); Fig. 5). To substantiate the observed HX effects, we also measured the rate of PNGase F-mediated deglycosylation of Asn-322(175) in the 170 loop of FVIIaM306D (Table 1). Deglycosylation of Asn-322(175) in FVIIaM306D proceeded significantly slower than in FVIIa, indicative of long-range coupling between the site of mutation at the TF interface and the 170 loop bordering the active site cleft. Protection from HX was also observed in the two /H9252-strands A2 (peptide 11: residues 275–287(129F-144); Fig. 4) and B2 (peptide 14: residues 297–302(155–160); Fig. 4) forming part of the activation pocket. In addition, the Ca2+/H11001-binding loop (peptide 5: residues 207–220(67–80); Fig. 4) of FVIIaM306D showed decreased HX. Remarkably, these reductions in HX are not reflected in the N-terminal tail of the FVIIaM306D variant. Rather, this part of the protease domain underwent slightly increased HX compared with wild-type FVIIa (peptide 1: residues 153–169(16–32); Fig. 4), and as shown in Table 1, the susceptibility of the N terminus of FVIIaM306D to carbamylation was indistinguishable from that of FVIIa. The other unique feature of the FVIIaM306D variant was seen in peptide 19 (residues 361–369(212–221A); supplemental Fig. 1b) covering the sequence immediately preceding activation loop 3. The HX of this segment in all the other variants, as well as in FVIIa, was unaffected upon TF binding but was clearly reduced in FVIIaM306D.

**Effect of TF Binding on the HX Profiles of FVIIa Variants**—Having measured the HX of the four free FVIIa variants, we performed HX-MS analyses of each variant in the presence of TF to compare their response to cofactor binding with that of the wild-type enzyme. Notably, the experiments were performed in large molar excess of TF (5-fold), and the TF binding affinities of the FVIIa variants are comparable to that of FVIIa (15–17). Overall, the binding of TF to any one of the four variants induced a reduction of HX resulting in a pattern highly similar to that of TF-bound FVIIa with a similar set (or subset) of peptides being affected (10) (Fig. 3a). However, as illustrated in Figs. 4 and 5 (right panels), several differences exist concerning the magnitude of reduction in HX upon TF binding. The N-terminal tail (peptide 1: residues 153–169(16–32); Fig. 4) in FVIIaVEAY, FVIIaDVQ, and FVIIaDVQA underwent reduced HX upon TF binding in a manner similar to that of TF-bound FVIIa, TF (right side). FVIIa data are denoted in the HX time-course plots by a dotted line. For clarity, FVIIa variant data are shown only if the deuterium content of a peptide deviates by more than 2 S.D. (0.4 deuteriums; see “Experimental Procedures”) from the deuterium content of the corresponding peptide in FVIIa. Altered intrinsic exchange (26) due to the mutation of FVIIaDVQ in the N-terminal tail (V158(21)D) could not account for the observed changes in deuterium incorporation of peptide 1 in FVIIaDVQA relative to FVIIa.
TABLE 2

Key regions of the FVIIa protease domain protected from HX in FVIIa variants

| Regiona | FVIIaM306D b | FVIIaVEAY | FVIIaDVQ | FVIIaDVQA |
|---------|--------------|-----------|---------|-----------|
| N-terminal tail (1) | ** | ** | ** | ** |
| Ca2⁺-binding loop (5) | * | * | *** | *** |
| β-Strand A2 (11) | *** | *** | *** | *** |
| β-Strand B2 (14) | * | * | ND | ND |
| 170 loop (16) | *** | *** | *** | *** |
| Activation loop 2 (18) | *** | *** | *** | *** |
| Activation loop 3 (20) | ** | ** | ** | ** |

* The peptides comprising the listed regions are indicated in parentheses.
† The N-terminal tail (peptide 1) of FVIIaM306D displayed slightly increased deuterium incorporation compared with FVIIa.

and the effect was even more pronounced for FVIIaDVQ and FVIIaDVQA. In contrast, the HX of the N-terminal tail of FVIIaM306D was completely unaffected by TF. The insensitivity of the N-terminal tail of FVIIaM306D was also evident from the inability of TF to protect the N terminus from carbamylation in this variant (Table 1). The TF-induced effects on the N-terminal tail were accurately mirrored in the neighboring Ca2⁺-binding loop (peptide 5: residues 207–220[67–80]; Fig. 4). TF binding to FVIIaVEAY and to a greater extent to FVIIaDVQ and FVIIaDVQA caused protection from HX similar to that seen with TF-bound FVIIa, whereas the HX of FVIIaM306D was unaffected by TF. This pattern of TF effects on the HX of the four variants was also observed, albeit less conspicuously, in the two β-strands A2 (peptide 11: residues 275–287[129F–144]; Fig. 4) and B2 (peptide 14: residues 297–302[155–160]; Fig. 4) of the activation pocket. The reduced effect on peptide 11 upon TF binding to FVIIaM306D might be the cause of the lack of an effect on its N-terminal tail peptide. Residues in peptide 11, in particular Arg-277[134] as well as Met-306[164], are crucial for TF to induce burial of the N terminus (32), and wrong conformation of this region might prevent insertion. In the 170 loop (peptide 16: residues 311–325[169–178]; Fig. 5), TF reduced the HX of the FVIIaVEAY variant below that of TF-bound FVIIa (by ~2 deuteriums). A similar but smaller effect was observed for TF-bound FVIIaM306D. Although the HX profiles of the TF-bound variants in activation loop 3 (peptide 20: residues 361–377[212–228]; Fig. 5) were largely similar (minor reductions for FVIIaVEAY and FVIIaDVQA to that of TF-bound FVIIa, a pronounced further reduction in HX was evident in several of the TF-bound variants, in particular FVIIaVEAY and FVIIaDVQA in activation loop 2 (peptide 18: residues 332–356[184–207]; Fig. 5).

DISCUSSION

A central challenge to understanding the activation and function of trypsin-like proteases is to pinpoint the roles played by key residues and regions and to trace the cross-talk between them. Here we used HX-MS to correlate the structural effects of mutations in FVIIa with distinctive functional consequences. Using four FVIIa variants, FVIIaM306D, FVIIaVEAY, FVIIaDVQ, and FVIIaDVQA, we assigned unique biochemical properties to distinct regions based on reduced HX (summarized in Table 2).
Analysis of the HX profiles in the framework of the HX effects observed in FVIIa upon TF binding allowed the observation of an intricate interplay between key sites in the protease domain.

**Met-306** is a Key Allosteric Trigger and Major Determinant of N-terminal Insertion—Mutations at position 306(164) in FVIIa were reported to reduce the activity enhancement normally caused by TF binding (15, 33). The original rationale behind the design of FVIIaM306D was that the M306(164)D mutation would stabilize the TF-binding helix and the active form of the protease domain and thereby induce an increase of intrinsic activity. However, FVIIaM306D failed in this respect, and the variant turned out not to respond to TF activation. Probing FVIIaM306D with HX-MS reveals surprising long-range effects on the conformation of the protease domain. As evident from Fig. 3, the mutation in the TF binding region reduces HX in a majority of peptides similarly affected by TF binding to FVIIa. The reduced rate of deglycosylation of Asn-322(175) verifies these effects in FVIIaM306D (Table 1). Apparently, despite having no effect on activity, the M306(164)D mutation does indeed stabilize the conformation of several regions within the protease domain and partially mimics the allosteric effect of TF, confirming position 306 as a key allosteric mediator in FVIIa. Why then does the M306(164)D mutation not render FVIIa more active? Most obviously, the conformational effects of the M306(164)D mutation do not encompass a reduction in the HX rates of the N-terminal tail as seen with TF-bound FVIIa and known to be critical for the activation of trypsin-like proteases (3, 4). Furthermore, the HX of FVIIaM306D, in peptides 18 and 19, covering activation loop 2 and a sequence preceding and including the beginning of activation loop 3, was significantly reduced to a level below that of TF-bound FVIIa (actually, peptide 19 of FVIIa was unaffected by TF binding). This indicates that the M306(164)D mutation introduces structural changes not observed in the TF-bound form of FVIIa. Because peptides 18 and 19 are linked by the disulfide bridge Cys-340(191)—Cys-368(220), we suggest that the dynamics or immediate environment of this disulfide is different in FVIIaM306D as compared with free FVIIa. This presumably results in a conformational change of Ala-369(221) in activation loop 3, which in the active form of FVIIa interacts with the inserted N terminus. The lack of stabilization of the inserted N-terminal tail might be a direct consequence of the unusual effects observed in activation loop 2 or 3 and the altered surroundings of the Cys-340(191)—Cys-368(220) disulfide. It is noteworthy that PABA is a similarly effective inhibitor of FVIIa and FVIIaM306D (Table 1), indicating that any alterations in the immediate environment of the Cys-340(191)—Cys-368(220) disulfide in FVIIaM306D are subtle in nature and do not perceptibly affect the S1 pocket. Surprisingly, the Ca$^{2+}$-binding loop, which has been shown to be structurally linked to the N terminus (10), displays similar HX in FVIIaM306D and TF-bound FVIIa. These observations suggest that part of the N-terminal tail is correctly aligned in FVIIaM306D, facilitating stabilization of the Ca$^{2+}$-binding loop via the Lys-157(20)—Val-299(157) main-chain hydrogen bond but that the N terminus as such (residues 153–156(16–19)) is not properly inserted or inserted at all. This is supported by carbamylation experiments with FVIIaM306D, showing indistinguishable exposure of the N terminus as compared with free FVIIa. The HX effects observed upon TF binding to FVIIaM306D show that the mutation severely abrogates the effect of TF; these observations are in agreement with carbamylation, deglycosylation, and PABA binding data (Table 1). The mere 2-fold stimulation of the amidolytic activity of FVIIaM306D by TF (15) might result from the minor conformational changes seen as partial reductions in HX rates in peptides 16 (170 loop), 20 (activation loop 3), 11 (β-strand A2), and 14 (β-strand B2) of FVIIaM306D upon TF binding. In conclusion, FVIIaM306D is inherently stabilized in several allosterically linked regions of the protease domain because of the M306(164)D mutation, but the very same mutation impairs normal allosteric activation by TF. These results confirm position 306 at the TF binding interface of the protease domain as a key allosteric site. The absence of N-terminal insertion in FVIIaM306D demonstrates the delicate interplay between the conformation of residue 306 and the transition of the protease domain into an active conformation with a correctly inserted N terminus.

**Origins of Enhanced Activity in FVIIa Variants**—FVIIaVEAY and FVIIaDVQ (as well as FVIIaDVQA) exhibit a dramatically enhanced intrinsic activity toward both peptidyl and macromolecular substrates. Although FVIIaVEAY, has the highest specificity constant for amidolytic activity, FVIIaDVQ displays the highest proteolytic activity. Interestingly, FVIIaVEAY and FVIIaDVQ exhibit different patterns of HX reduction in the protease domain relative to FVIIa. The moderate reductions in HX of FVIIaVEAY are largely confined to peptides covering the mutation sites (170 loop, activation loops 2 and 3), indicating localized effects in and around the active site region. The only other region that is affected by the mutations in FVIIaVEAY is β-strands A2 and B2 placed in the vicinity of activation loop 2 and with several interactions with the hinge regions of this loop. Strikingly, neither the N-terminal tail nor the Ca$^{2+}$-binding loop is affected, indicating that central elements of the “normal” allosteric activation of the protease domain are unaltered in this highly active variant. In contrast, FVIIaDVQ displays reduced HX in both the N-terminal tail and the Ca$^{2+}$-binding loop indicative of N-terminal insertion. The mutations in FVIIaDVQ are located in the N-terminal tail and β-strand B2, but reduced HX can be seen not only in integral parts of the activation pocket but also in activation loops 1 and 3 and to a lesser extent in activation loop 2. Evidently, N-terminal insertion facilitated by the mutations in FVIIaDVQ is perceived by several conformationally linked regions in the protease domain, in particular activation loops 1 and 3 and the Ca$^{2+}$-binding loop, giving the mutations an allosteric impact. The 170 loop and the 94 shunt do not appear to be directly linked to N-terminal insertion, as these regions show HX rates similar to FVIIa. A reduction in HX, indicative of conformational stabilization, in both of these loops appears absolutely to require TF binding or mutagenesis of other residues such as position 306 at the TF binding interface. Interestingly, from a comparison with FVIIaVEAY, N-terminal insertion and reduced HX in activation loop 1 and the Ca$^{2+}$-binding loop in FVIIaDVQ seem to correlate with its increased proteolytic activity. This implies that exosites for FX recognition and alignment are placed in the vicinity of the N-terminal tail and the Ca$^{2+}$-binding loop and that stabiliza-
tion of these regions facilitates the binding of FX to the protease domain of FVIIa. However, N-terminal insertion does not appear to influence the affinity of FX for FVIIa (34). In addition, FVIIaDVQ, despite its stably buried N terminus and dramatically enhanced proteolytic activity, displays at best a very modestly reduced $K_m$ for FX (17). The nature of residue 158$^{(21)}$ in the N-terminal tail appears to be critical in proteolytic activity regulation, with an Asp residue giving much higher activity than an Asn, while at the same time being without influence on the stability of the Ile-153$^{(16)}$—Asp-343$^{(194)}$ salt bridge (35). One should look upon the effects of the mutations in FVIIaDVQ as a shift in the equilibrium favoring the active conformation of FVIIa rather than as the creation of a new conformation because the exchange properties are within the spectrum limited by the extremes of free FVIIa and TF-bound FVIIa (even though the exact position differs between peptides). Only in complex with TF did we observe exchange kinetics slower than that of TF-bound FVIIa, indicating a local conformation in FVIIaDVQ never attained by FVIIa. This presumably reflects a local, mutational effect adding to the global stabilization conferred by TF. In other words, the few catalytically competent wild-type FVIIa molecules that are capable of cleaving FX are in the active conformation with an inserted N-terminal tail, but they constitute a small minority at any given point in time and drown in the pool of zymogen-like FVIIa molecules that determine the output from any analysis, including HX studies.

The different patterns of HX effects induced by the mutations in FVIIaVEAY and FVIIaDVQ are reflected in the HX behavior of FVIIaDVQ. This variant contains the K337$^{(188)}$A mutation also present in FVIIaVEAY and residing in activation loop 2 close to both the S1 pocket and Ser-344$^{(199)}$ of the catalytic triad. The HX rates of FVIIaDVQ are identical to those of FVIIaVEAY apart from a more pronounced reduction in the HX of activation loop 2, which correlates with the reduced HX observed in FVIIaVEAY in the same loop. Thus the K337$^{(188)}$A mutation induces a similar conformational effect on activation loop 2 regardless of the background. The extra reduction in HX of activation loop 2 is accompanied by a barely 2-fold increase in the activity of FVIIaDVQA compared with FVIIaDVQ.

Superimposition of the mutational effects on the structures of FVIIaDVQ and FVIIaVEAY shows that reduced HX of activation loop 3, $\beta$-strand A2, and $\beta$-strand B2 is a common feature of the variants with enhanced intrinsic activity. Despite these similarities, the two variants appear to gain improved catalytic efficiency by two different mechanisms (16). The HX profile of FVIIaVEAY shows that enhanced activity can be achieved without a facilitated N-terminal insertion and formation of the Ile-153$^{(16)}$—Asp-343$^{(194)}$ salt bridge. Instead, FVIIaVEAY attains enhanced activity by fine-tuning of the active site region through stabilization of the 170 loop and activation loops 2 and 3. In contrast, the mutations in FVIIaDVQ increase the propensity for insertion of the N-terminal tail into the activation pocket, which in turn produces an allosteric response partly mimicking the effect induced by TF. When we combine the HX analyses of the two highly active variants, the protease domain of FVIIa appears to have at least two key requirements for enhanced activity: N-terminal insertion and stabilization of the 170 loop. TF can induce both events, whereas the two completely different sets of mutations in FVIIaVEAY and FVIIaDVQ are able to reach only part of the way toward the fully active form of FVIIa. An FVIIa variant with the optimal mutations, able to induce conformational stabilization of the 170 loop and the same time facilitate N-terminal insertion without creating any conformational conflicts or clashes, would predictably have even higher intrinsic activity and a HX profile with closer resemblance to that of TF-bound FVIIa.

Based on intramolecular hydrophobic and hydrogen bond interactions connecting regions in the protease domain that undergo reduced HX upon either mutagenesis or TF binding, we suggest a model for the allosteric pathways in the protease domain of FVIIa (Fig. 6). The players in this dynamic network include the hydrogen bond between Arg-315$^{(170C)}$ and Gly-372$^{(223)}$ of the 170 loop and Gly-372$^{(223)}$ in activation loop 3 (25), the hydrophobic interaction between Leu-305$^{(163)}$ and Phe-374$^{(225)}$ of the TF-binding region and activation loop 3, respectively; II, the hydrophobic interaction between Leu-305$^{(163)}$ and Phe-374$^{(225)}$ of the TF-binding region and activation loop 3, respectively; III, the hydrophobic contacts between the 170 loop and the 94 shunt; IV, the extensive hydrophobic interactions between activation loop 2 and $\beta$-strands A2 and B2 of the activation pocket; V, the hydrophobic interaction between Ala-369$^{(221A)}$ and Val-154$^{(17)}$ of activation loop 3 and the N-terminal tail, respectively; VI, the salt bridge between Lys-161$^{(24)}$ and Asp-217$^{(77)}$/Asp-219$^{(79)}$ of the N-terminal tail and the Ca$^{2+}$-binding loop, respectively. The mutation sites are numbered and denoted by asterisks.

FIGURE 6. Long-range dynamic interplay in the protease domain of FVIIa. A schematic of the protease domain of FVIIa (adapted from the structure of the FVIIa-TF complex [7], Protein Data Bank code 1DAN) is shown with segments stabilized by mutagenesis (or TF binding) indicated in color (as in Fig. 3a). Denoted by yellow arrows are sites of hydrophobic or hydrogen bond interactions between regions undergoing reduced HX upon activation: I, the hydrophobic interaction between Arg-315$^{(170C)}$ and Gly-372$^{(223)}$ of the 170 loop and activation loop 3, respectively; II, the hydrophobic interaction between Leu-305$^{(163)}$ and Phe-374$^{(225)}$ of the TF-binding region and activation loop 3, respectively; III, the hydrophobic contacts between the 170 loop and the 94 shunt; IV, the extensive hydrophobic interactions between activation loop 2 and $\beta$-strands A2 and B2 of the activation pocket; V, the hydrophobic interaction between Ala-369$^{(221A)}$ and Val-154$^{(17)}$ of activation loop 3 and the N-terminal tail, respectively; VI, the salt bridge between Lys-161$^{(24)}$ and Asp-217$^{(77)}$/Asp-219$^{(79)}$ of the N-terminal tail and the Ca$^{2+}$-binding loop, respectively. The mutation sites are numbered and denoted by asterisks.
In summary, by monitoring HX rates we have probed the conformational dynamics of four FVIIa variants and shown that enhanced intrinsic activity can be attained by two different mechanisms. Mutations in certain regions of the protease domain can cause subtle effects that improve the local environment of the active site, whereas mutations of key residues can trigger an allosteric activation of the protease domain emulating the effect of the physiological cofactor TF. Analysis of the conformational effects induced by site-directed mutagenesis revealed an intricate network of cross-talk between key sites in the protease domain, providing details pertinent for the normal allosteric pathway of FVIIa.

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