Incorporation of an Active Site Inhibitor in Factor VIIa Alters the Affinity for Tissue Factor*

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Recent studies showed that the administration of active site-inhibited factor VIIa blocked factor VIIa/tissue factor-induced fibrin and thrombus formation in ex vivo and in vivo model systems. These studies suggest that inactivated factor VIIa competes efficiently with plasma factor VII(a) for a limited number of tissue factor sites. In the present study, we compared the interactions of factor VIIa and active site-inhibited factor VIIa with tissue factor. Competition studies of factor VIIa and active site-inhibited factor VIIa in a factor X activation assay showed that the affinity of the latter for relipidated tissue factor was 5-fold higher than that of factor VIIa. Radioligand binding studies with a human bladder carcinoma cell line (J82) and surface plasmon resonance studies using soluble tissue factor demonstrated a faster association and a slower dissociation for the active site-inhibited factor VIIa. Studies of equilibrium binding to cell surface tissue factor showed that the affinity of active site-inhibited VIIa was 5-fold higher than that of factor VIIa to non-functional tissue factor sites, whereas both inactivated factor VIIa and factor VIIa bound to functional tissue factor sites with the same high affinity. Comparison of the CD spectra of factor VIIa and active site-inactivated factor VIIa revealed structural differences in the protease domain. The potential physiological implications of these findings are discussed.

The in vivo initiation of the coagulation cascade is triggered by the binding of plasma factor VIIa (FVIIa) to the cell surface receptor tissue factor (TF) (1). TF is normally expressed in adventitial cells and pericytes surrounding blood vessels but not in cells that come in contact with blood, such as monocytes and endothelial cells (2, 3). Tissue injury disrupting the endothelial cell barrier is normally required for FVIIa to come in contact with TF. However, under pathological conditions monocytes and endothelial cells could be perturbed to induce TF.

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† The abbreviations used are: FVIIa, activated coagulation factor VII; FFR-FVIIa, d-Phe-l-Phe-l-Arg methylene FVIIa; des(1–44)-FFR-FVIIa, FFR-FVIIa lacking the N-terminal 44 amino acids; des(1–44)-FFR-FVIIa, FFR-FVIIa lacking the N-terminal 44 amino acids; TF, tissue factor; sTF, soluble extracellular portion of tissue factor (residues 1–219); FX, coagulation factor X; FXa, activated FX; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

(4–7)

Factor VII circulates as a single chain zymogen, and the binding to TF markedly increases the susceptibility of factor VII to cleavage at Arg152 resulting in formation of a two-chain serine protease, FVIIa. TF is a glycoprotein consisting of 263 amino acids with a 219-amino acid extracellular part. The extracellular part is structured in two fibronectin type III-like domains (8, 9). The crystal structure of d-Phe-l-Phe-l-Arg (FFR)-FVIIa-soluble TF (sTF) complex has recently been determined (10). In this complex, FVIIa has been shown to adopt an extended conformation and wrap around TF with the Gla domain near the cell membrane and the catalytic domain distal to it.

Administration of inactivated FVIIa was shown to reduce angiographic restenosis and decrease neointimal hyperplasia in a rabbit atherosclerotic injury model (11) and also to reduce thrombus formation at sites of vascular injury in a baboon femoral balloon artery angioplasty model (12). In a preliminary study, it was shown that infusion of a low concentration of inactivated FVIIa inhibited the endotoxin-induced drop in platelets and fibrinogen levels and abolished the deposition of fibrin in kidneys in a rabbit model (13). Additional studies in an ex vivo model (14, 15) and in vitro (16) also showed that TF-induced coagulation and thrombogenesis could be effectively blocked by inactivated FVIIa. The low concentration of inactivated FVIIa required for blocking coagulation in some of these studies (11, 13) raised the possibility that inactivated FVIIa may have a higher affinity than plasma FVIIa for TF.

The present study was carried out to compare the interaction of inactivated FVIIa with TF to that of native FVIIa. Inactivated FVIIa (FFR-FVIIa) was prepared by incorporating the peptide chloromethyl ketone d-Phe-l-Phe-l-Arg in the active site of FVIIa. The interaction of FFR-FVIIa and FVIIa with TF was analyzed by radioligand binding assays using a cell line that constitutively expressed cell surface TF and also by a biospecific interaction analysis as quantified by surface plasmon resonance. Further, the inhibitory effect of FFR-FVIIa on FVIIa/TF-catalyzed activation of factor X (FX) was analyzed using both a cell-based system and in suspension with relipidated TF. CD measurements showed subtle differences between FFR-FVIIa and FVIIa structure. Overall, our data were consistent with the hypothesis that blocking of the FVIIa active site enhanced the affinity for TF.

EXPERIMENTAL PROCEDURES

Cell Culture—A human bladder carcinoma cell line (J82) was obtained from American Type Culture Collection (ATCC: HTB-1). The cell line was grown in T-75 flasks and subcultured into 24-well tissue culture plates as described (17). The cell number at confluency was about 175,000 cells/well.

Proteins—Recombinant human FVIIa was isolated as described (18). The active site of recombinant FVIIa was blocked by addition of a 2-fold molar excess of d-Phe-l-Phe-l-Arg chloromethyl ketone (Bachem). The
solution was incubated for 1 h at 4 °C. Unreacted n-Phe-n-Phe-n-Arg chloromethyl ketone was separated from active site-blocked FVIIa by Q-Sepharose Fast Flow ion exchange chromatography, a purification step that was used in the final stage of purification of FVIIa. FFR-FVIIa was eluted with 10 mM CaCl2, and the profile of the eluted peak was narrow and symmetrical. The residual FVIIa activity in FFR-FVIIa was <0.1% when measured in an FVIIa-specific amidolytic assay. Des-(1–44)-FVIIa and des-(1–44)-FFR-FVIIa (19), factors X (FX) (20) and Xa (Fxa) (21), human brain TF apoprotein (22), sTF (23), and polyclonal rabbit anti-human TF IgG (3) were prepared as described previously. TF apoprotein was reconstituted into 60% phosphatidylcholine, 40% phosphatidylethanolamine vesicles as described (25).

Radiolabeling of Proteins—FVIIa and FFR-FVIIa were labeled using IODO-GEN (Pierce)-coated tubes and Na125I according to the manufacturer's technical bulletin and as described previously (26). The labeling reaction was performed in tubes coated with 10 μg of IODO-GEN for 4 min on ice. The reaction was quenched by the addition of KI (1%), and free iodine was removed by extensive dialysis against 10 mM Hepes, pH 7.5, 150 mM NaCl. The concentration of the labeled proteins was determined by A280 measurements.

FVIIa Binding to Cell Surface TF—Binding studies employing J82 cells were carried out as described (26). Briefly, confluent monolayers in 24-well tissue culture plates were washed once with Buffer A (10 mM Hepes, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) supplemented with 1% SDS and 5 mM EDTA and then washed twice with Buffer A. In experiments to measure binding at equilibrium, the monolayers were incubated for 2 h at 4 °C with varying concentrations of radiolabeled FVIIa or FFR-FVIIa in Buffer B (Buffer A supplemented with 1 mg/ml bovine serum albumin and 5 mM CaCl2) in a final volume of 300 μl. In experiments measuring the association rates of FVIIa and FFR-FVIIa binding, the monolayers were incubated at 37 °C with a fixed concentration of radiolabeled FVIIa or FFR-FVIIa for varying time periods. At the end of the incubation, the monolayers were washed 4 times with ice-cold Buffer B and lysed with lysis buffer (200 mM NaOH, 1% SDS, 10 mM EDTA). Radioactivity was measured in a γ counter (Cobra, Packard Instrument Co.). For all experiments, nonspecific binding was determined in parallel duplicate wells in which the monolayers were preincubated for 15 min with rabbit anti-human TF IgG (100 μg/ml) before the addition of radioligand. TF-specific binding was determined by subtracting nonspecific binding from total binding. The binding data were analyzed and curve-fitted using GraFit (Erithacus Software, Ltd., Staines, Middlesex, United Kingdom) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Dissociation of cell surface TF-bound FVIIa and FFR-FVIIa was determined essentially as described earlier (27).

**FX Activation Assay on J82 Cells**—Confluent cell monolayers in a 24-well plate were incubated with FVIIa or a combination of FVIIa and FFR-FVIIa in Buffer B (final volume, 300 μl) at 37 °C for 30 min to allow for the saturation of ligand binding to the cell surface TF. Unbound ligand was then removed, and the monolayers were washed 4 times with ice-cold Buffer B. The activation of FX was initiated by overlaying the monolayers with 300 μl of Buffer B containing 100 nM FX. After 20 min, 20-μl aliquots were removed from each well and added to 80 μl of stopping buffer (50 mM Tris, pH 7.5, 150 mM NaCl, containing 5 mM EDTA and 1 mg/ml bovine serum albumin). The amount of FXa generated was determined in a chromogenic assay by transferring 50 μl of the above mixture to a microtiter plate well and adding 50 μl of Chromozym X (1.25 mg/ml) to the well. The absorbance at 405 nm was measured continuously in a microplate reader (Molecular Devices), and the initial rates of color development were converted to FXa concentrations using an FXa standard curve (85 pM–11 nM) in a microtiter plate well. For all experiments to measure binding at equilibrium, the monolayers were incubated for 2 h at 4 °C with varying concentrations of radiolabeled FVIIa or FFR-FVIIa in Buffer B (final volume, 300 μl). In experiments measuring the association rates of FVIIa and FFR-FVIIa binding, the monolayers were incubated at 37 °C with a fixed concentration of radiolabeled FVIIa or FFR-FVIIa for varying time periods. At the end of the incubation, the monolayers were washed 4 times with ice-cold Buffer B and lysed with lysis buffer (200 mM NaOH, 1% SDS, 10 mM EDTA). Radioactivity measured in a γ counter (Cobra, Packard Instrument Co.). For all experiments, nonspecific binding was determined in parallel duplicate wells in which the monolayers were preincubated for 15 min with rabbit anti-human TF IgG (100 μg/ml) before the addition of radioligand. TF-specific binding was determined by subtracting nonspecific binding from total binding. The binding data were analyzed and curve-fitted using GraFit (Erithacus Software, Ltd., Staines, Middlesex, United Kingdom) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

**CD Spectroscopy**—CD spectra of FVIIa and FFR-FVIIa and their des-(1–44) forms were acquired in 10 mM borate, pH 7.5, 5 mM CaCl2 as described (23) using a Mark5 (Jobin-Yvon Instruments SA) spectrophotometer. The final protein concentration was 0.5–0.7 mg/ml. The instrument was calibrated using 10-camphorsulfonic acid at 290 nm, and each CD spectrum represents the average of three scans obtained by collecting data at 0.5-nm intervals with an integration time of 2 s. In the far-UV region, a 0.1-mm path length was used, whereas a 5-mm path length was used in the near-UV region.

**RESULTS**

Binding of FVIIa and FFR-FVIIa to Relipidated TF—Binding of FVIIa and FFR-FVIIa to relipidated TF was examined by measuring FVIIa-TF functional activity. In an initial experiment, various concentrations of FVIIa (0.01–1.25 nM) were incubated with reconstituted TF (0.05 nM), and the expression of FVIIa-TF functional activity was measured in a FX activation assay. Increasing concentrations of FVIIa up to 0.2 nM yielded increasing rates of FX activation (data not shown). A concentration of 0.1 nM FVIIa gave 80–90% of the maximal rate of FX activation. The binding of FFR-FVIIa to TF was analyzed as the ability of FFR-FVIIa to inhibit FVIIa-TF-catalyzed activation of FX using various concentrations of FFR-FVIIa in the presence of 0.1 nM FVIIa. In a reverse experiment, the ability of FVIIa to overcome the inhibition by FFR-FVIIa was examined by adding increasing concentrations of FVIIa in the presence of 0.1 nM FFR-FVIIa. The results (Fig. 1) showed that half-maximal inhibition in the presence of 0.1 nM FVIIa was obtained at a concentration of 0.045 ± 0.012 nM FFR-FVIIa. In contrast, a 5-fold higher concentration of FVIIa (0.28 ± 0.05 nM) was required to restore 50% of the maximal activity in the presence of 0.1 nM FFR-FVIIa. Since these data suggested that FFR-FVIIa bound to TF with a higher affinity than FVIIa, we carried out further experiments to investigate the binding of FFR-FVIIa to cell surface TF.

**Time Course of 125I-FVIIa and 125I-FFR-FVIIa Binding to Cell Surface TF**—Confluent monolayers of J82 cells were incubated with 10 nM 125I-FVIIa or 125I-FFR-FVIIa at 37 °C for varying times, and the binding of radioligand to cell surface TF was determined. In agreement with earlier binding studies (17, 26), the data showed that FVIIa binding to cell surface TF was time-dependent and required about an hour to reach maximum binding (Fig. 2). The data also showed a faster association of FFR-FVIIa to cell surface TF, and only 15–20 min were re-
required to reach maximal binding. Since FVIIa and FFR-FVIIa were labeled by identical procedures, the difference between FVIIa and FFR-FVIIa in the rate of binding to cell surface TF could not be due to differences in radiolabeling. Further, measurements of functional activity of cell-bound 125I-FVIIa showed a similar rate of FX activation as that obtained with unlabeled FVIIa.

Dissociation of 125I-FVIIa and 125I-FFR-FVIIa Bound to Cell Surface TF—Monolayers of J82 cells were incubated with 10 nM 125I-FVIIa or 125I-FFR-FVIIa at 37 °C for 60 min. The cells were washed to remove free ligand and were overlaid with Buffer B containing 500 nM unlabeled ligand to avoid rebinding of the dissociated labeled ligands to the cell surface. At different time points, the supernatant was removed and its radioactivity was measured. As shown in Fig. 3, the cell surface FVIIa binding had a dissociation half-time of 27 ± 5 min in accordance with the dissociation half-time (31 ± 6 min) reported for FVIIa bound to cell surface TF of OC-2008 (27). In contrast, FFR-FVIIa bound to cell surface TF dissociated at a slower rate with a dissociation half-time of 53 ± 5 min. Since both the on- and off-rates indicated a higher affinity of FFR-FVIIa for cell surface TF, we have further characterized the binding under equilibrium conditions.

Binding Characteristics of 125I-FVIIa and 125I-FFR-FVIIa to Cell Surface TF—Monolayers of J82 cells were incubated with varying concentrations of 125I-FVIIa and 125I-FFR-FVIIa for 120 min at 4 °C to reach equilibrium binding, and the amount of cell-bound radioactivity was determined. As shown in Fig. 4A, both FVIIa and FFR-FVIIa bound to cell surface TF in a concentration-dependent manner and approached saturation. However, saturation was reached at a lower concentration with FFR-FVIIa than with FVIIa. When the saturation binding data were transformed into a Scatchard plot (Fig. 4B), a single binding site for FFR-FVIIa with a $K_d$ of 2.7 ± 0.9 nM and a maximum binding ($B_{\text{max}}$) of 86 ± 22 fmol/well (295,000 ± 75,000 sites/cell) (n = 4) was found, whereas the data for FVIIa suggested a two-site model with $K_{d,\text{high}}$ of 2.0 ± 0.6 nM and $B_{\text{max,high}}$ of 34 ± 8 fmol/well (n = 4). The low affinity binding site was characterized by a $K_{d,\text{low}}$ of 12 ± 6 nM and a $B_{\text{max,low}}$ of 64 ± 41 fmol/well. The total number of binding sites for FVIIa (98 ± 46 fmol/well (336,000 ± 158,000 sites/cell)) was similar to that of FFR-FVIIa.

Factor Xa Generation on Monolayers of J82 Cells in the Presence of FVIIa and FFR-FVIIa—To further characterize FVIIa and FFR-FVIIa binding to cell surface TF, we measured rates of FX activation at various concentrations of FVIIa and FFR-FVIIa.

FFR-FVIIa. A dose-response curve of FVIIa showed that a concentration of 1 nM FVIIa yielded 80–90% of maximal activity, and this concentration was used in the subsequent competition studies. FVIIa (1 nM) was added to the cells together with various concentrations of FFR-FVIIa (Fig. 5). The concentration of FFR-FVIIa needed to reduce the FVIIa-dependent FX activation by 50% (the IC$_{50}$ value) was 1.1 ± 0.4 nM. When the cells were incubated with a fixed concentration of 1 nM FFR-FVIIa and varying concentrations of FVIIa, we obtained an EC$_{50}$ value (the concentration of FVIIa needed to restore 50% of maximal activity) of 1.0 ± 0.2 nM. The IC$_{50}$ and EC$_{50}$ values
were not significantly different, indicating equal affinities of FVIIa and FFR-FVIIa for functional TF sites.

**Biosensor Measurements of Interactions of FVIIa and FFR-FVIIa with sTF—**Characterization of protein-protein interactions in real time and in the absence of phospholipids was performed using surface plasmon resonance (Fig. 6). The data showed approximately 1.5-fold faster association and 3-fold slower dissociation of FFR-FVIIa compared with FVIIa. The calculated dissociation half-times based on the obtained dissociation rates were 16 ± 1 min for FVIIa and 50 ± 4 min for FFR-FVIIa, thus corroborating the results obtained using J82 cells. The resultant $K_d$ values were approximately 0.6 nM for FFR-FVIIa and 3 nM for FVIIa. Similar results were obtained using three different batches of each ligand at two different concentrations of each ligand (10 and 30 nM).

**CD Spectroscopy of FVIIa and FFR-FVIIa—**Far-UV CD spectra of FVIIa and FFR-FVIIa and their corresponding des-(1–44) forms (Fig. 7) were similar to the previously published spectra of FVIIa (23). However, a few minor differences were found between the FVIIa and FFR-FVIIa spectra. FFR-FVIIa yielded higher amplitudes of the ellipticity maximum and minimum (Fig. 7A). A slight red shift of the maximum was seen with FFR-FVIIa, whereas the position of the minimum was unaltered. Similar minor differences were also observed when comparing the des-(1–44) forms of FVIIa and FFR-FVIIa (Fig. 7B). This suggests that the secondary structural differences are in the protease domain. The near-UV spectra showed an increased signal between 250 and 300 nm after inactivation of FVIIa both in the presence and absence of residues 1–44 (Fig. 8). Since the light chain of FVIIa has been shown not to contribute to the near-UV CD spectrum of FVIIa (23), this indicates that changes in the tertiary structure affecting the environment of Tyr residues have occurred in the protease domain as a result of incorporation of the inhibitor.

**DISCUSSION**

The data presented here provide convincing evidence that the incorporation of a small peptide inhibitor in the active site of FVIIa enhances its affinity for TF. In the present study, the direct binding of FVIIa and FFR-FVIIa to TF was characterized by two different methods: (i) by measuring the binding parameters of radiolabeled FVIIa and FFR-FVIIa to monolayers of J82 cells that constitutively express cell surface TF, and (ii) by monitoring the binding of FVIIa and FFR-FVIIa to sTF using a BIAcore instrument. In both systems, we obtained very similar results, i.e. FFR-FVIIa associated with TF at a faster rate and dissociated from it at a slower rate compared with FVIIa. The results also showed that FVIIa bound to TF with high affinity, and this was further increased with the incorporation of the active site inhibitor.

Equilibrium binding studies of FVIIa and FFR-FVIIa to the cell surface TF also revealed differences between FVIIa and FFR-FVIIa binding to TF. The binding data obtained with FFR-FVIIa revealed a single class of binding sites with a $K_d$ value of 2.7 nM. In contrast, the binding data obtained with FVIIa was best fitted to a two-site model with $K_d$ values of 2.0 nM and 12 nM, respectively. Since both FVIIa and FFR-VIIa binding curves are slightly curvilinear on the Scatchard plot and the downward deflection of the Scatchard plot of FFR-FVIIa could mean that the binding of FFR-FVIIa to cell surface TF may be cooperative, the Hill equation was fit to the data. The goodness of fit of the binding curves to the Hill model was
similar to that of hyperbola curve fitting. Further, the Hill coefficient for both FFR-FVIIa and FVIIa was close to 1.0 (0.95 for FFR-FVIIa and 0.85 for FVIIa), suggesting that neither FVIIa nor FFR-VIIa binding to cell surface TF was cooperative. The present binding data of FVIIa to J82 cell surface TF differ from that of previous studies of FVIIa binding to this cell type. Le et al. (26) hypothesized in an earlier study that two distinct FVIIa binding sites exist on these cells: a minor population of “functional” sites and a major population of “non-functional” sites. Although Scatchard analysis of their binding data did not fit well to a two-site binding model, the studies on the expression of FVIIa-TF functional activity provided suggestive evidence for the existence of two differing TF sites. The binding data of FVIIa of the present study (which showed factor VIIa bound to a minor fraction of TF sites with a higher affinity than it bound to a major fraction of TF sites) are consistent with the above hypothesis. If so, the binding data obtained with FFR-FVIIa could be interpreted as if FFR-FVIIa binds to both functional and non-functional TF sites with the same high affinity. However, it should be pointed out that we have no direct evidence that indicates that the high affinity binding represents the formation of a functional FVIIa-TF complex.

In conclusion, our data showed that the incorporation of a small peptide inhibitor in the active site of FVIIa enhanced its binding data from each and every experiment were best fitted with a two-site model. At present, we do not know the reason for this discrepancy between our data and the data of Sakai et al. (17). It is possible that we detected two classes of binding sites because we used a wider range of FVIIa concentrations than employed in the previous study. Nevertheless, it should be pointed out that the number of total FVIIa binding sites determined in the present study (336,000 sites/cell) was very similar to that reported in the earlier study (320,000 sites/cell) (17).

To explain the discrepancy between FVIIa binding and the expression of FVIIa-TF functional activity on OC-2008 cells, Fair and MacDonald (29) observed a sigmoidal factor VII binding curve with an apparent \( K_d \) of 314 pm and 38,000 binding sites/cell. Further, Hill plots of their data revealed a single slope of 2.1, suggesting positive cooperativity with a minimum of two binding sites for Factor VII. A positive cooperativity was also observed in FVIIa binding to TF in reconstituted phospholipid vesicles (30). The degree of cooperativity depended on the vesicle charge and the form of the enzyme. One possible reason for differences between our binding data and those reported by Fair and MacDonald (29) could be due to a significant difference in the way the binding assays were performed. In our binding studies, monolayers were washed with calcium-containing buffer, whereas a calcium-free buffer was used by Fair and MacDonald (29). It was shown that about 70–80% of cell-associated FVIIa was consistently eluted when monolayers of J82 cells were washed with a buffer not containing calcium (17). Differences in phospholipid composition at the vicinity of TF and other possible differences between cell surface TF and purified TF could explain why our FVIIa binding data differed from that obtained with relipidated TF (30).

Since our FVIIa binding data on J82 monolayers also differ somewhat from the data of Sakai et al. (17), who reported a single class of binding sites for FVIIa, we have repeated the experiment multiple times (\( n = 6 \), four at 4 °C and two at 37 °C), and each experiment was analyzed independently. Although the binding data of some of these experiments could be fitted reasonably well to a single class of binding sites, the...
affinity for TF. This could explain why administration of a low concentration of inactivated FVIIa functioned as an effective anticoagulant and reduced TF-induced thrombus formation. The ability of FFR-FVIIa to bind to non-functional TF with the same affinity as it binds to functional TF would lead to a situation in which the majority of non-functional TF are complexed with FFR-VIIa. Such FFR-FVIIa\textsubscript{z} TF complexes could limit the formation of functional VIIa\textsubscript{z}TF complexes even after the TF-bearing cells were altered by injury or inflammatory stimuli in a manner that would normally transform non-functional TF to functional TF.

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Page 11863, right-hand column, line 11: The word confirmation should be corrected to read conformation.

Page 11864, Fig. 1, legend title: The words FFR-FVIIa-FVIIa' TF should be corrected to read FFR-FVIIa in FVIIa' TF.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.