Isolation and Characterization of Thrombin-activated Human Factor VIII*

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Recombinant human factor VIII (FVIII) was activated by thrombin at pH 7.4, followed by CM-Sepharose chromatography at pH values ranging from 3.5 to 7.4. Optimal coagulant activity was recovered at pH 5.5 and was associated with the isolation of an A1/A2/A3-C1-C2 heterotrimer. The activity was stable at ~80 °C, but decayed slowly (t1/2 ~1 week) and nonproteolytically at room temperature or 4 °C. The coagulant activity of the pH 5.5 FVIII preparation assayed in human hemophilia A plasma was only 20% that of porcine factor VIIIa. However, its activity was approximately 75% that of porcine FVIIIa in a plasma-free assay, indicating that human FVIIIa is unstable relative to porcine FVIIIa during the coagulation assay. The first-order rate constant for spontaneous, nonproteolytic loss of activity of human FVIIIa at pH 7.4 was decreased 5-fold by fIXa and phospholipid, indicating that human FVIIIa is stabilized when incorporated into the intrinsic pathway factor X activation complex.

Activated factor VIII (fVIIIa) is a cofactor for membrane-dependent proteolytic activation of FIX by fIXa (fIXa). Thrombin-activated FVIII is a 160-kDa heterotrimer composed of A1, A2, and A3-C1-C2 subunits (1-3). The activation of human FVIII at its physiological concentration (~1 μM) at pH 7.4 is followed by first-order loss of activity (t1/2 = 2 min) (4-10). This process is accompanied by dissociation of the A2 subunit and does not result from further proteolytic modification (5, 7, 10, 11).

Structure-function studies of FVIIIa have been hampered by its lack of stability and consequent difficulties associated with its isolation. Porcine FVIIIa has been isolated by monoclonal fast protein liquid chromatography at pH 6.0 and is indefinitely stable when stored at concentrations exceeding 0.2 μM (1). However, several attempts to isolate human FVIIIa by this or other procedures have not been successful because of dissociation of the A2 subunit and recovery of little or no heterotrimeric FVIIIa (2, 3, 12, 13). Whether all of the properties of porcine FVIII and FVIIIa are shared by human FVIII and FVIIIa has been questioned (14, 15). For example, the decay rate of porcine FVIIIa is decreased by approximately 10-fold when it is bound to fIXa on a phospholipid surface (7-9). However, fXa cleaves and inactivates human FVIIIa (15, 16), and the notion that human FVIIIa is stabilized by fIXa, while supported by one study (17), has been challenged (15). In none of these studies has the stabilization of FVIIIa by fIXa been directly tested by using purified forms. Additionally, it has been proposed that human FVIII has less coagulant activity than porcine FVIII because of more rapid loss of its A2 subunit (2, 10), but comparison of purified human and porcine FVIIIa in coagulation assays has not been possible. In this study, the isolation of active, heterotrimeric human FVIIIa and its comparison to porcine FVIIIa is described.

EXPERIMENTAL PROCEDURES

Materials—Human thrombin (1800 units/mg) was obtained from Baxter Biotech, Hyland Division (Glendale, CA). Normal human plasma and hemophilia A plasma were purchased from George King Biomedical. n-Phenylalanil-polygly-arginyl chloromethyl ketone was purchased from Calbiochem. Methoxycarbonyl-n-cyclohexylglycyl-glycyl-arginine-p-nitroanilide (Spectrozyme Xa) was purchased from American Diagnostica. Fluorescein-5-maleimide and succinimidyl-(acetyldithio)acetate were purchased from Molecular Probes. Small unilamellar phosphatidylcholine/phosphatidylserine (PC/PS) (70/30, w/w) vesicles were prepared as described previously (18).

Protein Isolation—Published procedures were used to isolate porcine factors IXa, X, and Xa (7, 19) and porcine FVIII and FVIIIa (1). Human plasma-derived FVIII was purchased from Cutter Biologicals. Human fIXa was purchased from Enzyme Research Laboratories (South Bend, IN). Porcine fIXa modified at the active site with 5-(dimethylamino)-1-naphthalenesulfonylglutamyl-arginy1-CH2Cl (DEGR-fIXa) was prepared as described previously (8). Fluorescein-maleimide-thioacetyl-FPR-fIXa (FM-FPR-fIXa) was prepared as described previously (20, 21).

Isolation of Human FVIIIa—All procedures were carried out at room temperature unless otherwise indicated. The starting material for the purification of human FVIIIa was bulk human recombinant FVIII obtained from Baxter Biotech Group, Hyland Division. FVIII (0.4 mg/ml) in 0.4 M NaCl, 20 mM Tris-Cl, 5 mM CaCl2, pH 7.4, 0.01% (w/v) Tween 20, pH 7.4, was first dialyzed into 0.15 M NaCl, 20 mM Hepes, 5 mM CaCl2 overnight at 4 °C and then stored at ~20 °C until further use. FVIII (3.1 μM, 10 ml) was activated by human thrombin (0.1 μM) for 10 min at 37 °C. Activation was stopped by addition of the thrombin inhibitor FPR-CH2Cl (0.3 μM) along with 10 ml of 100 mM sodium acetate buffer, at pH 5.5. The sample was loaded at 2 ml/min onto a CM-Sepharose column (1 × 10 cm) equilibrated in 0.1 M NaCl, 25 mM sodium acetate, 5 mM CaCl2, 0.01% Tween 20, pH 5.5. FVIIIa was eluted at 0.5 ml/min using a 30-ml 0.1-1.0 M NaCl gradient in the same buffer. Fractions containing FVIIIa were collected and immediately adjusted to pH 6.0 by addition of 0.5 M Mes.

Extinction Coefficients and Molecular Weights—Extinction coefficients (ε280) at 280 nm and molecular masses used were: porcine FVIIIa, 1.90, 160 kDa (1); porcine fIXa, 1.62, 45 kDa (7); porcine fX, 1.04, 57 kDa (7). The extinction coefficients of human FVIII and FVIIIa were assumed to be the same as porcine FVIIIa.

Coagulation Assays—FVIIIa was measured by using human FVIII-deficient plasma as a substrate as described previously (22). The coagulant activity was determined in reference to the standard one-stage
fVIII assay in which 1 unit of fVIII is defined as the amount of fVIII in 1 ml of normal human plasma.

**Defined (Plasma-free) Assay of fVIIIa**—A chromogenic substrate assay for fVIIIa was used in which FX was activated by an enzymatic complex consisting of FXa, PCPS, calcium, and limiting amounts of fVIIa. Under the conditions used, the initial velocity of FX activation is directly proportional to the concentration of fVIIIa.

**Fluorescence Measurements**—The binding of fVIIa to FXa was studied by measuring the increase in fluorescence anisotropy of FI-M-FPR-FXa which occurs upon binding fVIIa. Measurements were made at a normal plasma concentration of FX at 25 °C using a Jasco 710 spectropolarimeter. Samples were dialyzed overnight at 4 °C into 0.1 M NaCl, 0.02 M Heps, 2 mM CaCl₂, pH 7.4, containing 19 nM FI-M-FPR-FXa, 100 μM PCPS, and variable concentrations of fVIIIa as described previously.

**Circular Dichroism Spectroscopy**—Far-ultraviolet CD spectra of human fVIII and several other proteins were measured. The CD spectra of human fVIIIa were observed below 200 nm. The observed difference is consistent with less random structure.

**RESULTS**

**Isolation of Human fVIIIa**—Attempts to isolate active human fVIIIa by mono-S chromatography at pH ranging from 4.5 to 7.4 were not successful. We then attempted to purify human fVIIIa by using CM-Sepharose to lower the charge density of the column matrix. Several pH conditions ranging from 4.5 to 7.4 were tested (Fig. 1). At pH 3.5, very little protein was recovered. At pH 4.5–4.7, near the pKₐ of the CM-Sepharose carboxymethyl group, the A1 subunit was eluted as a separate peak prior to a A2/A3-C1-C2 dimer, as judged by SDS-PAGE analysis (Fig. 2). At the other pH extreme, pH 7.4, the A2 subunit dissociated from the A1/A3-C1-C2 dimer. Isolation of the A1/A2/A3-C1-C2 heterotrimer was optimal between pH 5.0 and 6.0 as judged by the amount of A1 and A2 subunits detected by SDS-PAGE (Fig. 2).

The specific coagulant activity of fVIIIa isolated at the various pH conditions tested was maximal at pH 5.0–5.5 and decreased markedly at lower and higher pH values (not shown). The activity of fVIIIa isolated at all pH values decreased very slowly relative to the decay of activity of fVIII activated in situ at the normal plasma concentration of fVIII (≈1 nM) at pH 7.4 (t₀½ = 2 min) (10). However, these results are in contrast to the behavior of isolated porcine fVIIIa, which is indefinitely stable at 4 °C or room temperature at pH 6.0 at concentrations exceeding 0.2 μM (1).

The slow loss of activity of the pH 5.5 preparation (t₀½ = 6 days) was not decreased by p-phenylalanyl-prolyl-arginyl chloromethyl ketone or hirudin. After several weeks at 4 °C, a minor band at 25 kDa was observed whose formation was inhibited by benzamidine. Thus, the slow loss of activity appears to be predominantly nonproteolytic with an additional minor loss due to proteolysis. Since many oligomeric proteins undergo denaturation at 4 °C but not room temperature (25), the pH 5.5 preparation was also evaluated at room temperature, but was not more stable. fVIIIa was indefinitely stable when stored at −80 °C.

**Circular Dichroism Spectra of Human fVIII and fVIIIa**—The far-ultraviolet CD spectra of human fVIII and several preparations of human fVIIIa are shown in Fig. 3. The CD spectra of the different pH preparations of human fVIIIa are indistinguishable, suggesting that a pH-dependent loss in coagulant activity is not due to gross differences in the secondary structure of fVIIIa.
coil structure in human FVIIIa, presumably due to the removal of the thrombin-cleaved B-domain by CM-Sepharose chromatography. The increase in the negative CD band at 218 nm may also indicate an increase of β-sheet structure in FVIIIa formed from FVIII.

Coagulant Activity of Human FVIIIa—The coagulant activities of human FVIIIa preparations isolated at pH 4.7 and 5.5 were compared to porcine FVIIIa (Table I). The specific activity of human FVIIIa was constant over at least a 10-fold concentration range, corresponding to clotting times of 55–65 s. The determinations made to construct Table I correspond to clotting times in this range.

The specific activity of human FVIIIa isolated at pH 5.5 (130,000 units/mg, 21,000 units/nmol) is similar to the specific coagulant activity produced at the peak of thrombin activation of human FVIII (80,000–110,000 units/mg, 17,000–23,000 units/nmol) (2, 3), suggesting that the isolated FVIIIa is fully functional. However, the coagulant activity of the human pH 5.5 preparation was approximately 6-fold lower than that of porcine FVIIIa. Possibly, human FVIIIa decays faster than porcine FVIIIa during the performance of coagulation assay, i.e. from the time that FVIIIa is diluted to the time of visible clot formation. At the concentrations used in the coagulation assays, the half-lives of human FVIIIa and porcine FVIIIa activities under these conditions are 2 and 6 min, respectively (10). These decay times are on the same scale as the time necessary to produce the end point of the coagulation assay, about 1 min. Thus, the relatively greater loss of activity of human FVIIIa could lead to a lower observed specific activity, even if both human and porcine FVIIIa were fully functional prior to dilution into the assay system.

Stabilization of Human FVIIIa by FIXa and Phospholipid—The decay of activity of FVIIIa can be measured using a defined plasma-free assay (10). The relative activities of human and porcine FVIIIa can then be compared without the potential complications of undetermined loss of activity during the assay. Before comparing human and porcine FVIIIa in a defined system, the effect of FIXa on FVIIIa was evaluated, since it has been reported that FIXa proteolytically inactivates human FVIIIa (15, 16), whereas FIXa stabilizes porcine FVIIIa (7). In the absence of FIXa and phospholipid, the half-lives of human and porcine FVIIIa were approximately 2 and 6 min, respectively (Fig. 4). Addition of FIXa and phospholipid increased the half-life of human FVIIIa to 12 min and porcine FIXa to 60 min.

The stabilization of porcine FVIIIa by FIXa or DEGR-FIXa was also compared. We examined the possibility that DEGR-FIXa, which is active site-blocked and has no detectable enzymatic activity, might stabilize FVIIIa to a greater extent than FIXa. Fig. 4 shows that DEGR-FIXa produced a 1.7-fold longer half-life than FIXa (100 versus 60 min). SDS-PAGE analysis of the porcine FVIIIa after incubation revealed the appearance of a minor band migrating between the A1 and A2 fragments when FIXa but not DEGR-FIXa was present (not shown). This band has similar electrophoretic behavior to that described during cleavage of human FVIII and FVIIIa by FIXa (15, 16) and identified as derived from the A1 domain, presumably due to cleavage at Arg<sup>20</sup>. Thus, stabilization of factor VIIIa by FIXa and inactivation of factor VIIIa by FIXa are competing processes. At the concentration of FIXa used in Fig. 4 (10 nM), FVIIIa is nearly saturated with FIXa (20), representing optimal stabilization. As

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**Table I**

| FVIIIa preparation | Specific activity<sup>a</sup> | Percent relative to porcine FVIIIa activity<sup>c</sup> |
|--------------------|-----------------------------|---------------------------------------------------|
| Porcine, pH 6.0    | 630,000 ± 110,000           | 50                                                |
| Human, pH 4.7      | 31,000 ± 4,000              | 75                                                |
| Human, pH 5.5      | 130,000 ± 30,000            | 50                                                |

<sup>a</sup> Human or porcine FVIII were activated by thrombin at pH 7.4 and then FVIIIa was isolated by CM-Sepharose or Mono-S chromatography, respectively, at the indicated pH.

<sup>b</sup> Data are expressed as the mean ± 1 S.D. for four to eight determinations.

<sup>c</sup> Human or porcine FVIIIa samples were diluted 20,000–100,000-fold to adjust the measured clotting times to 55–65 s.

<sup>d</sup> Percent activity was determined by extrapolation to zero time of initial velocity curves.

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**Fig. 3.** Circular dichroism spectra of human FVIII and human FVIIIa. CD spectra were measured as described under “Experimental Procedures.” FVIII (▲); FVIIIa, pH 4.7 (●); FVIIIa, pH 5.0 (□); FVIIIa, pH 5.5 (●); FVIIIa, pH 6.0 (○).

**Fig. 4.** Stabilization of human and porcine FVIIIa by FIXa and phospholipid. Human FVIIIa (2 nm), in the presence (▲) or absence (○) of human FIXa (10 nM) and PC/PS (20 μM), was incubated for the indicated times in 0.15 M NaCl, 0.02 M Hepes, 5 mM CaCl<sub>2</sub>, 0.01% Tween 80, pH 7.4 (Buffer A). FVIIIa activity was measured by diluting the mixture to 200 μl into the same buffer containing additionally 20 nm human FIXa and 20 μM PC/PS, followed by addition of porcine FIX to 300 nm and determination of the initial velocity of FX activation as described under “Experimental Procedures.” Porcine FVIIIa in the presence (▲) and absence (○) of porcine FIXa was measured in the same way except that the final concentration of porcine FVIIIa in the FX activation solution was 50 pm. Additionally, stabilization of porcine FVIIIa by porcine DEGR-FIXa (20 nm) and PC/PS (20 μM) was evaluated exactly as for porcine FIXa (▼). The data are expressed as the fraction remaining relative to initial FVIIIa (FVIII<sub>A</sub>), which was calculated by extrapolation of a semi-log plot to zero time.
the concentration of fXa increases, the inactivation reaction would become more prominent.

Comparison of Human and Porcine FVIIIa Activity in a Defined System—In contrast to the coagulation assay, where possible loss of FVIIIa activity cannot be evaluated, FVIIIa activity can be followed directly in the plasma-free assay. In this assay, the activity of the pH 5.5 human FVIIIa preparation was 75% that of porcine FVIIIa (not shown). This result is in contrast to a relative activity of approximately 20% when compared to the coagulation assay (Table I). When human FVIIIa was isolated at pH 4.7, the specific activity of the preparation in the plasma-free assay was considerably lower than the pH 5.5 preparation (not shown), consistent with results obtained with the coagulation assay (Table I).

Human and porcine FVIIIa were also compared by activating highly purified FVIII with thrombin and comparing initial velocities of IX activation in the plasma-free assay. At the concentration of thrombin used, FVIIIa was activated within 1 s, which allows comparison of peak activation of FVIII and subsequent decay rates. Both plasma-derived and recombinant human FVIII were compared to porcine FVIII and gave similar results (Fig. 5). In the porcine system, the initial rates of IX activation when FVIII is activated in situ at 200 pm are very similar to the values obtained by using purified FVIIIa at the same concentration, indicating that no major changes in activity of FVIIIa occur during the process of separating FVIIIa from activation peptides and thrombin. Human FVIII was activated to levels that were indistinguishable from porcine FVIII. Comparison of the results shown in Fig. 3 to those obtained with FVIIIa preparations (Table I) indicates isolation of human FVIIIa at pH 5.5 yields a preparation that approaches, but may not achieve, complete activity.

Binding of Human FVIIIa and Porcine FVIIIa to fIXa—Porcine FVIIIa preparations appear completely active since a stoichiometry of 1 FVIIIa molecule per fIXa molecule is obtained by fluorometric titration (20). These titrations are done by measuring the increase in fluorescence anisotropy of FI-M-FPR-fIXa, a derivative of fIXa in which a single fluorescein molecule is covalently attached near the active site. FI-M-FPR-fIXa undergoes a large increase in fluorescence anisotropy (r) when saturated with porcine FVIIIa in the presence of phospholipid (Δr = 0.09). The increase in anisotropy of a fixed concentration of FI-M-FPR-fIXa as a function of FVIIIa concentration can be used to determine the stoichiometry and the apparent dissociation constant of the porcine FVIIIa/fIXa interaction.

The titration of porcine FI-M-FPR-fIXa by the pH 4.7 and 5.5 human FVIIIa preparations was studied fluorometrically and compared to porcine FVIIIa (Fig. 6). The stoichiometry and apparent dissociation constant for porcine FVIIIa (Table II) was similar to values previously reported (20). The anisotropy increase produced by the two human FVIIIa preparations correlated with the measured activity. Both human FVIIIa preparations produced lower anisotropy increases than porcine FVIIIa. The human preparations yielded apparent stoichiometry val-
uses greater than 1. If these high stoichiometry values are due to the presence of inactive forms, this would lead to an underestimation of the true concentration of active FVIII and an overestimation of the dissociation constant. The higher dissociation constants could also arise from weaker binding of human FVIII to porcine F1-M-FPR-fXa.

**DISCUSSION**

The assembly of the intrinsic pathway IX activator complex is essential for normal hemostasis. Difficulties associated with the isolation of a stable form of FVIII have hampered the analysis of this complex. Activation of porcine FVIII by thrombin followed by Mono-S high pressure liquid chromatography at pH 6 results in the isolation of a A1/A2/A3/C1-C2 heterotrimer with indefinitely stable activity at 4 °C (1). Porcine FVIII isolated in this way appears completely active because it binds fXa with 1:1 stoichiometry (20) and molecular weight analysis by analytical ultracentrifugation is consistent with a homogeneous population of heterotrimers (1). However, attempts to isolate human FVIIIa by Mono-S HPLC in this study and others (2, 3, 13) were unsuccessful, instead yielding mainly the inactive Al/A3-Cl-C2 dimer. We now find that active, heterotrimERIC human MIIa can be isolated by CM-Sepharose chromatography at pH 5.5. This procedure constitutes the first report in which the A2 subunit of human FVIIIa is recovered in high yield.

The properties of this human FVIIIa preparation were characterized in several ways and used to address controversial or unresolved issues in the analysis of FVIIIa structure and function. Compared to porcine FVIIIa, the coagulant activity of human FVIIIa preparations is low (Table I). However, this appears to be at least partly due to the relative instability of human FVIIIa during the assay itself (i.e. in plasma during the development of the fibrin clot) since the discrepancy is much lower when a plasma-free assay is used for comparison (Table I). Inactivation of FVIIIa during the plasma-free assay can be monitored and is slow relative to the assay period. The activity of the pH 5.5 human FVIIIa preparation approaches that of porcine FVIIIa in this assay and is similar to that obtained by the peak activation of human FVIII by thrombin. In the coagulation assay, the activity of this preparation (130,000 units/mg) is similar to the peak coagulant activity generated by the activation of FVIII by thrombin (2, 3). The increase in fluorescence anisotropy of F1-M-FPR-fXa produced by this preparation of human FVIIIa yields an apparent stoichiometry for the FVIIIa-fXa interaction slightly greater than 1 (Table II). These results suggest that human FVIIIa isolated in this way is nearly completely active, and provides a suitable reagent for analysis of intrinsic pathway activation of fX in the human system.

In contrast to porcine FVIIIa, human FVIIIa was not indefinitely stable at 4 °C or room temperature. Although benzamidine slightly decreased the rate of decay and prevented the appearance of a 25-kDa proteolytic fragment, most of the loss of activity was not correlated with a proteolytic event. This non-proteolytic loss of activity is distinct from the nonproteolytic decay in activity that occurs over a period of a few minutes at pH 7.4 at nanomolar concentrations of FVIIIa due to dissociation of the A2 subunit. This slow loss of activity occurred at FVIIIa concentrations much greater than the dissociation constant for the binding of the A2 subunit to the A1/A3-C1-C2 dimer at pH 6.0 (28 nM) (26). Thus, the loss of activity appears to be due to a secondary pathway accessible to human FVIIIa, possibly irreversible denaturation of the heterotrimer or of the A2 subunit while free in solution. Storage at –80 °C is a practical way to maintain active FVIIIa preparations for further characterization.

The pH 5.5 human FVIIIa preparation was used to address a controversial issue regarding the lack of stability of FVIIIa. Although porcine FVIIIa is stabilized by fIXa and phospholipid, it has been proposed that this is not the case with human FVIIIa because fXa cleaves and inactivates human FVIIIa (15). However, the first-order decay of human FVIIIa activity is decreased 8-fold by nanomolar concentrations of fIXa and saturating concentrations of phospholipid (Fig. 4). This result was compared to the stabilization of porcine FVIIIa, since previous studies measured porcine FVIIIa inactivation mixtures that contained thrombin and FVIII activation peptides. Purified porcine FVIIIa, like purified human FVIIIa, is stabilized by fXa and phospholipid (Fig. 4), indicating that neither the activation peptides nor thrombin contribute to the stabilization process. As in the case of human FVIIIa, fXa cleaves porcine FVIIIa, apparently in the A1 domain at Arg<sup>356</sup>. This cleavage either inactivates or reduces the activity of porcine FVIIIa, because the stabilization of FVIIIa by active site-blocked fXa is greater than with active fXa (Fig. 4). However, the dominant effect of fXa at nanomolar concentrations is the stabilization of FVIIIa.

The behavior of human FVIIIa in the coagulation assay has implications regarding the measurement of FVIII in the clinical setting. The FVIIIa assay used in this study utilizes the same reagents as the standard one-stage FVIII clinical assay. In the latter assay, FVIIIa is activated endogenously by feedback from thrombin (27). Porcine FVIIIa has higher specific coagulant activity than human FVIIIa (10), suggesting either a difference in the rate of FVIIIa activation or in the stability of FVIIIa. By studying the coagulant activity of purified human FVIIIa and thus eliminating consideration of the activation process, the stability of FVIIIa appears to be a critical determinant of coagulant activity.

The simplest mechanism consistent with this result is that after FVIIIa is diluted into plasma, substantial dissociation of the A2 subunit and loss of FVIIIa activity occurs during the 55–65 s required for the formation of a visible fibrin clot. The A2 subunit is in reversible equilibrium with the A1/A3-C1-C2 dimer (10, 17, 26). The dissociation constants for human and porcine FVIIIa are similar at pH 7.4, approximately 0.2–0.3 μM, which is much higher than the concentration of FVIII in plasma or in clotting assays. Thus, in dilute solutions of FVIIIa, activity is governed entirely by the dissociation rate constant. The dissociation rate constant of human FVIIIa at pH 7.4 is 3-fold higher than porcine FVIIIa (0.35 min<sup>−1</sup> versus 0.12 min<sup>−1</sup>) (10) which may explain the difference in behavior between human and porcine FVIII and FVIIIa in coagulation assays. Additionally, unidentified factors in human plasma may exist that further destabilize human FVIIIa relative to porcine FVIIIa.

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**TABLE II**

| FVIIIa preparation | $K_d$ | $n^a$ | $\Delta n_m$<sup>b</sup> |
|--------------------|-------|-------|--------------------------|
| Porcine            | 3.4 ± 0.7 | 0.8 ± 0.1 | 0.085 ± 0.002 |
| Human, pH 5.5      | 23 ± 11  | 17 ± 0.6  | 0.073 ± 0.002 |
| Human, pH 4.7      | 29 ± 11  | 29 ± 0.8  | 0.072 ± 0.002 |

<sup>a</sup> Moles of FVIIIa bound per mole of F1-M-FPR-fXa (apparent).

<sup>b</sup> Increase in fluorescence anisotropy of F1-M-FPR-fXa saturated with FVIIIa; parameters are expressed as the mean ± 1 S.D.
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