McEver, R. P., Bennett, E. M., & Martin, M. N. (1983) *J. Biol. Chem. 258*, 5269-5275.
Montgomery, R. R., Kunicki, T. J., Taves, C., Pidard, D., & Corcoran, M. (1983) *J. Clin. Invest. 71*, 385-389.
Mustard, J. F., Perry, D. W., Ardlie, N. G., & Packham, M. A. (1972) *Br. J. Haematol. 22*, 193-204.
Newman, J., Johnson, A. J., Karpatkin, M. H., & Puszkin, S. (1971) *Br. J. Haematol. 21*, 1-20.
Niewiarowski, S., Budzynski, A. Z., Morinelli, T. A., Brudzynski, T. M., & Stewart, G. J. (1981) *J. Biol. Chem. 256*, 917-925.
Ouyang, C., & Huang, T.-F. (1983) *Biochim. Biophys. Acta 757*, 332-341.
Peerschke, E. I. B. (1985) *Semin. Hematol. 22*, 241-259.
Phillips, D. R., Charo, I. F., Parise, L. V., & Fitzgerald, L. A. (1988) *Blood 71*, 831-843.
Pidard, D., Montgomery, R. R., Bennett, J. S., & Kunicki, T. J. (1983) *J. Biol. Chem. 258*, 12582-12586.
Plow, E. F., & Marguerie, G. A. (1980) *Blood 56*, 553-555.
Plow, E. F., & Ginsberg, M. H. (1981) *J. Biol. Chem. 256*, 9477-9482.
Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., & Ginsberg, M. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8057-8061.
Poncz, M., Eisman, R., Heidenreich, R., Silver, S. M., Vilaire, G., Surrey, S., Schwartz, E., & Bennett, J. S. (1987) *J. Biol. Chem. 262*, 8476-8482.
Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. R., & Ruoslahti, E. (1986); *Science (Washington, D.C.) 231*, 1559-1562.
Ruggeri, Z. M., Demarco, L., Gatti, L., Bader, R., & Montgomery, R. R. (1984) *J. Clin. Invest. 72*, 1-12.
Ruoslahti, E., & Pierschbacher, M. D. (1987) *Science (Washington, D.C.) 238*, 491-497.
Shattil, S. J., Hoxie, J. A., Cunningham, M., & Brass, L. F. (1985) *J. Biol. Chem. 260*, 11107-11114.
Shelton-Inloes, B. B., Titani, K., & Sadler, J. E. (1986) *Biochemistry 25*, 3164-3171.
Switzer, M. E., & McKee, P. S. (1976) *J. Clin. Invest. 57*, 925-937.
Timmons, S., Kloczewiak, M., & Hawiger, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4935-4939.
Titani, K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., & Fujikawa, K. (1986) *Biochemistry 25*, 3171-3184.
Williams, S., & Gralnick, H. (1987) *Thromb. Res. 46*, 457-471.

Subunit Structure of Thrombin-Activated Porcine Factor VIII†

Pete Lollar*,‡,§ and Carlo G. Parker‡
Departments of Medicine and Biochemistry, University of Vermont, Burlington, Vermont 05405
Received May 23, 1988; Revised Manuscript Received August 11, 1988

ABSTRACT: Factor VIII (fVIII) is synthesized as a single chain having a domainal sequence A1-A2-B-A3-C1-C2. Analysis of the proteolyic cleavage of fVIII by thrombin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) identifies three fragments designated $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ with fragment(s) derived from the B domain being difficult to visualize. The appearance of these fragments is associated with the development of coagulant activity, but the activity is labile without further apparent proteolysis. In this study, porcine fVIII was reacted with thrombin until peak coagulant activity was obtained and then subjected to cation-exchange (Mono S) high-pressure liquid chromatography. Coagulant activity was recovered in a single peak that contained all three fragments and was stable for weeks at 20 °C in 0.65 M NaCl/0.01 M His-HCl/0.005 M $CaCl_2$ at pH 6.0. Analytical ultracentrifugation of activated fVIII was done to test whether all three fragments were associated. The apparent molecular weight of activated fVIII from equilibrium sedimentation increased from 148 000 to 161 000 as the loading concentration was increased from 0.06 to 0.16 mg/mL. This agrees well with the summed apparent molecular weights of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ calculated from SDS–PAGE analysis (148 000) or from the amino acid sequence of human fVIII (159 000). This establishes the major species in the preparation as a $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ heterotrimer and additionally indicates either weak self-association of the trimer and/or incomplete association of the individual subunits to form the trimer. Velocity sedimentation of activated fVIII revealed a single boundary ($s^0_{20,w}$ = 7.2 S). From the combined velocity and equilibrium sedimentation data, a frictional coefficient ratio of 1.39 was calculated, indicating that activated fVIII is moderately asymmetrical.

Factor VIII (fVIII),[1] the plasma protein that is decreased or absent in patients with hemophilia A, has been the subject of extensive biochemical and clinical investigation for over 50 years. It circulates tightly bound in a noncovalent complex with von Willebrand factor (vWf). Recent advances have

Table I: Yield of $FVIIIa_{IIa}$ from Mono S Chromatography[a]

detergent[b]	expts	fractional yield[c]	specific activity[d]
yes	6	0.57 ± 0.10[e]	7000 ± 800[e]
no	2	0.58	8100

[a] The yield ranged from 0.5 to 2.2 nmol (0.07–0.33 mg). [b] 0.01% Tween 80. [c] Mole of $fVIIIa_{IIa}$ recovered per mole of fVIII in activation mixture. [d] $FVIIIa_{IIa}$ coagulant activity (units per nanomole). [e] Mean ± SD.

† This work was supported by an American Heart Association Established Investigator Award (P.L.), by a grant-in-aid from the American Heart Association with funds contributed in part by the Vermont affiliate, and by the Vermont SCOR in Thrombosis (HL-35058).
* Address correspondence to this author at the Department of Medicine, University of Vermont, Burlington, VT 05405.
‡ Department of Medicine.
§ Department of Biochemistry.

0006-2960/89/0428-0666$01.50/0

included the isolation of fVIII from bovine (Vehar & Davie, 1980), porcine (Fass et al., 1982), and human (Fulcher & Zimmerman, 1982) plasma and the molecular cloning of the fVIII gene (Toole et al., 1984; Wood et al., 1984; Gitschier et al., 1984; Vehar et al., 1984). It is isolated as a mixture of calcium-linked heterodimers (Fass et al., 1982) due to a proteolytic cleavage between the B and A3 domains and variable proteolysis in the B domain. This results in a C-terminal-derived light chain and a variably sized heavy chain, which contains the A1 and A2 domains but lacks some or all of the B domain. FVIII requires proteolytic activation to participate fully as a cofactor in the activation of factor X by factor IXa in the intrinsic pathway of blood coagulation. Thrombin and factor Xa have been the most studied activators and catalyze distinct proteolytic pathways (Lollar et al., 1985; Eaton et al., 1986a). In the process of forming $fVIIIa_{IIa}$, thrombin cleaves fVIII at arginine-372 between the A1 and A2 domains, at arginine-740 between the A2 and B domains, and at arginine-1689, 41 residues from the N-terminus of the light chain (Vehar et al., 1984; Toole et al., 1984; Eaton et al., 1986a), and thereby produces three stainable fragments, $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$. Following the activation of fVIII, many studies have found that a substantial loss of activity occurs over a period of about 1 h. The reason for the instability of $fVIIIa_{IIa}$ remains obscure. Because of the labile nature of $fVIIIa_{IIa}$, its subunit structure has been difficult to determine. We now find that porcine $fVIIIa_{IIa}$ isolated from thrombin and fVIII activation fragments is stable and consists of a $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ heterotrimer.

Experimental Procedures

Materials. Activated partial thromboplastin reagent was purchased from General Diagnostics (Morris Plains, NJ). FVIII-deficient plasma was purchased from George King Biomedical, Inc. (Overland Park, KS). Sephadex G25 superfine (fast desalting column), Mono S, and Mono Q (HR 5/5) chromatography resins were purchased from Pharmacia (Piscataway, NJ). The W3-3 hybridoma cell line producing mouse monoclonal anti-porcine fVIII was generously provided by Dr. D. N. Fass, Mayo Clinic, Rochester, MN. Porcine fVIII concentrate was purchased from Porton Products (Encino, CA). Human recombinant fVIII was a generous gift from Dr. Dan Eaton, Genentech, Inc., South San Francisco, CA.

Isolation of Proteins. All steps were done at room temperature unless otherwise indicated. FVIII was isolated by using monoclonal anti-fVIII immunoaffinity chromatography as previously reported (Lollar et al., 1988a) and was stored in the column elution buffer [1 M NaCl/5 mM His-HCl/2.5 mM $CaCl_2$/50% (v/v) ethylene glycol] at 4 °C before use. The monoclonal antibody is specific for $fVIII_{LC}$ (Fass et al., 1982). FVIII was further fractionated by 1/4 dilution into 0.04 M Tris-HCl/5 mM $CaCl_2$/0.01% (v/v) Tween-80 at pH 7.4 and application to a Mono Q HR 5/5 column equilibrated in 0.15 M NaCl/0.02 M Tris-HCl/5 mM $CaCl_2$/0.01% Tween 80 at pH 7.4 at a flow rate of 2 mL/min. FVIII was eluted with a 20-mL linear 0.15–0.75 M NaCl gradient in this buffer at a flow rate of 1 mL/min. Two peaks emerged from the Mono Q column as judged by absorbance at 280 nm. The first peak, at 0.4 M NaCl, was identified as $fVIII_{LC}$ by SDS–PAGE, represented 10–20% of the protein applied to the column, and contained no activity. FVIII coagulant activity was in the second peak, at 0.55 M NaCl, which usually was pooled for further use. The shoulder of the second peak was enriched in a species of fVIII ($fVIII_{A1\text{-}A2/LC}$), which lacks the B domain as reported previously for Mono Q chromatography of human fVIII (Andersson et al., 1986; Fay et al., 1986). Fractions containing only $fVIII_{A1\text{-}A2/LC}$ were used for some experiments. The average specific activity of the pooled fVIII from five preparations was 840 units/mg. All preparations could be activated 40–60-fold by thrombin in a two-stage clotting assay. The specific activity of porcine fVIII is approximately 10-fold lower than that reported for human fVIII, which appears to be due to the use of porcine plasma as a standard in the assay (Lollar et al., 1988a).

To isolate $fVIIIa_{IIa}$, all fractions from the Mono Q column containing fVIII coagulant activity were pooled and diluted 1/3 with 0.01 M Tris-HCl/5 mM $CaCl_2$/0.01% Tween 80 at pH 7.4 to lower the ionic strength to 0.2. The resulting concentration of fVIII ranged from 0.2 to 0.4 μM. Porcine thrombin, prepared as described previously (Lollar et al., 1984), was added to a final concentration of 0.035 μM, and the reaction mixture was assayed for clotting activity at 2-min intervals until peak coagulant activity was reached, followed by the addition of PPACK to a final concentration of 0.15 μM. The reaction mixture was diluted 1/2 into 0.03 M His-HCl/5 mM $CaCl_2$/0.01% Tween 80 at pH 6.0 and applied to a Mono S HR 5/5 column equilibrated in 0.1 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$/0.01% Tween 80 at pH 6.0 at 2 mL/min. After washing the column with this buffer until the absorbance at 280 nm was less than 0.003, $fVIIIa_{IIa}$ was eluted with a 20-mL linear NaCl gradient from 0.1 to 0.75 M in the same buffer at a flow rate of 1 mL/min. In some experiments, Tween 80 was omitted from the gradient buffers (see below). Two peaks emerged as judged by absorbance at 280 nm. An initial, minor peak at 0.38 M NaCl was identified as PPA-thrombin by SDS–PAGE (Figure 2, lane 2). The second peak emerged at 0.65 M NaCl and contained the coagulant activity. $fVIIIa_{IIa}$ was stored in the elution buffer at room temperature or at 4 °C for further analysis.

The thrombin-cleaved light chain of porcine fVIII ($fVIII_{A3\text{-}C1\text{-}C2}$) was prepared as described previously (Lollar et al., 1988b).

Assays. FVIII and $fVIIIa_{IIa}$ were measured by one-stage and two-stage assays, respectively, in an activated partial

[1] Abbreviations: vWF, von Willebrand factor; PPACK, D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone; PPA-thrombin, D-phenylalanyl-L-prolyl-L-arginylthrombin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography. The following nomenclature is used regarding factor VIII (fVIII). Amino acid residues are numbered according to the sequence derived from human fVIII cDNA (Vehar et al., 1984). $FVIIIa_{IIa}$ refers to the molecular species responsible for the activity in a clotting (Fass et al., 1982) or chromogenic substrate (Lollar et al., 1985) assay that occurs upon exposure of fVIII to thrombin (factor IIa). The term fragment is used to designate a single-chain polypeptide derived from proteolytic cleavage of single-chain fVIII. The term subunit is used when there is physical evidence for the association of fragments of fVIII. The fVIII light chain, $fVIII_{LC}$, is the subunit of fVIII containing residues 1649–2332 of single-chain fVIII. Some fragments of fVIII are named relative to the dominal sequence A1-A2-B-A3-C1-C2 of fVIII (Vehar et al., 1984; Eaton et al., 1986a,b) to avoid the variable nomenclature that results from naming fragments by their apparent molecular weights. $FVIII_{A1}$ is the fragment generated by thrombin that contains the entire A1 domain and residues 1–372. $FVIII_{A2}$ is the fragment generated by thrombin that contains the entire A2 domain and appears to contain residues 373–740. $FVIII_{A1\text{-}A2}$ is the fragment that has not been cleaved between the A1 and A2 domains by thrombin and contains residues 1–740. $FVIII_{A3\text{-}C1\text{-}C2}$ is the fragment generated from $fVIII_{LC}$ by thrombin or factor Xa that contains the entire A3, C1, and C2 domains (residues 1690–2332). Noncovalent subunit associations are denoted by a (/) whereas covalent associations are denoted by a (–). For example, $fVIII_{A1\text{-}A2/LC}$ denotes a heterodimer consisting of fragments $fVIII_{A1\text{-}A2}$ and $fVIII_{LC}$.

thromboplastin time assay using human fVIII deficient plasma as a substrate (Fass et al., 1982). One unit of porcine fVIII was defined as the amount of fVIII in 1 mL of normal citrated porcine plasma.

Sedimentation Analysis. Experiments were done by using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and a multiplexer. The optical system included a cylindrical lens and mirrored optics and was interfaced to a microcomputer with an Isaac Model 41A data acquisition system (Cyborg Corp., Newton, MA). Protein was measured by absorbance at 280 nm. Sample cells contained a 12-mm double-sector centerpiece and sapphire windows. All runs were made in a solution of fVIII containing 0.65 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$ at pH 6.0. The solvent sector contained HPLC column buffers mixed to give a NaCl concentration of 0.65 M. Runs were made with the temperature controller off, which resulted in a constant temperature between 16 and 20 °C.

Equilibrium sedimentation was done by using the high-speed meniscus depletion technique (Yphantis, 1964). Samples (0.1 mL) underwent centrifugation for 36 h in an An-F four-place rotor at 13 000 rpm prior to data acquisition. The rotor was increased to 15 000 rpm for 24 h prior to additional data acquisition. Then the rotor was accelerated to 52 000 rpm and maintained at that speed for 30 min prior to deceleration back to 15 000 and further data acquisition. This allowed for an estimate of the base-line absorbance. All absorbance values from the meniscus to the bottom of the cell, for a total of approximately 60 data points, were included in the analysis. The data were fit by nonlinear least-squares analysis using the Marquardt algorithm (Bevington, 1969) to the equation describing the distribution of a single species at sedimentation equilibrium:

$$A_d = A(r_o) \exp[\sigma/2(r^2 - r_o^2)] + A_b$$

where A_d is the observed digitized absorbance plus base line, $A(r_o)$ the absorbance at some reference radius r_o in the cell, r the radial distance, A_b the digital base line, and σ the effective reduced molecular weight (Yphantis, 1964). Three parameters σ, $A(r_o)$, and A_b were optimized in the fitting procedure. Estimated standard deviations associated with σ were less than 2% of the value of σ in all runs and were independent of the value selected for r_o. The apparent molecular weight M_r(app) was calculated by using the relationship

$$\sigma = M(1 - \bar{v}\rho)\omega^2/RT$$

where M is the molecular weight, $\bar{v}$ the partial specific volume of the protein, ρ the solution density, ω the rotor angular velocity, R the ideal gas constant, the T the absolute temperature. The partial specific volume was calculated from the amino acid composition (Cohn & Edsall, 1943) deduced from the human fVIII cDNA sequence (Vehar et al., 1984; Toole et al., 1984), assuming the presence of three fragments corresponding to positions 1–372 ($fVIII_{A1}$), 373–740 ($fVIII_{A2}$), and 1690–2332 ($fVIII_{A3\text{-}C1\text{-}C}$) in single-chain fVIII. A correction for carbohydrate due to the five potential asparagine-linked glycosylation sites in the three fragments was not made. Solution density was estimated from the known sodium chloride concentration and tabulated values for solutions of sodium chloride.

Sedimentation velocity runs were done on 0.3-mL samples in an An-D rotor at 60 000 rpm. Scans were obtained at 4-min intervals. The sedimentation coefficients of the protein in the Mono S purified $fVIIIa_{IIa}$ preparation and of $fVIII_{A3\text{-}C1\text{-}C2}$ were determined by measuring the equivalent boundary position by the method of second moments and corrected for solvent density, viscosity, and temperature (Cantor & Schimmel, 1980a). The frictional coefficient f of $fVIIIa_{IIa}$ and that of an equivalent anhydrous sphere f_{min} were calculated as described (Cantor & Schimmel, 1980b).

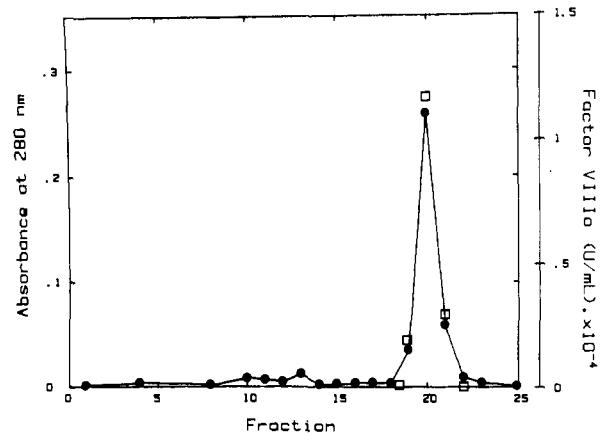

FIGURE 1: Mono S chromatography of $fVIIIa_{IIa}$. FVIII (0.25 μM) in 0.17 M NaCl/0.015 M Tris-HCl/5 mM $CaCl_2$/0.01% Tween 80 at pH 7.4 was reacted with 0.035 μM thrombin for 7 min and divided into two samples, followed by the addition of PPACK (0.15 μM) to one of the samples. An aliquot containing 0.32 mg of fVIII of the sample containing PPACK immediately underwent HPLC using a Mono S HR 5/5 resin as described under Experimental Procedures. Fractions (1 mL) were assayed for absorbance at 280 nm (circles) and $fVIIIa_{IIa}$ (squares).

Extinction Coefficients. The following values were used for published extinction coefficients ($E_{1cm}^{0.1\%}$) at 280 nm: porcine thrombin, 1.99 (Lollar et al., 1984); porcine fVIII, 1.2 (Lollar et al., 1988a). The extinction coefficient for $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ was determined to be 1.6 according to the ultraviolet spectroscopy method of van Iersel et al. (1985).

Electrophoresis. SDS–PAGE was done by using the buffer system of Laemmli (1970) under conditions described previously (Lollar et al., 1987), followed by silver staining of the proteins (Morrissey, 1981).

Results

Isolation of Thrombin-Activated FVIII ($FVIIIa_{IIa}$). FVIII was reacted with thrombin and either subjected to HPLC after the development of peak coagulant activity or allowed to remain in contact with thrombin. An irreversible inhibitor of thrombin, PPACK, was added to the sample prior to the HPLC step. A separate control sample was included that did not contain PPACK or undergo HPLC. FVIII coagulant activity emerged as a single symmetrical peak from the HPLC column at a sodium chloride concentration of 0.65 M (Figure 1). SDS–PAGE of the peak fraction (Figure 2, lane 1) and the other fractions containing activity showed three bands corresponding to $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$. Additionally, a small peak due to PPA-thrombin emerged at approximately 0.4 M NaCl (Figure 2, lane 2). Control experiments showed that PPA-thrombin emerged from the column at a lower ionic strength than thrombin and that PPACK did not bind the resin. Therefore, PPACK was included in the fVIII activation mixture to avoid contamination of purified $fVIIIa_{IIa}$ by thrombin. An additional band migrating between the bands at 76 and 130 kDa was seen during the activation of fVIII that did not appear in the HPLC-purified material (Figure 2, lanes 4 and 5). The origin of this band has not been identified but may represent a large B-domain fragment.

In Figure 3, kinetics of activation of fVIII corresponding to the HPLC experiment are shown. In the absence of the HPLC step, most of the activity disappeared within 1 h and continued to decrease over the next 24 h. The addition of

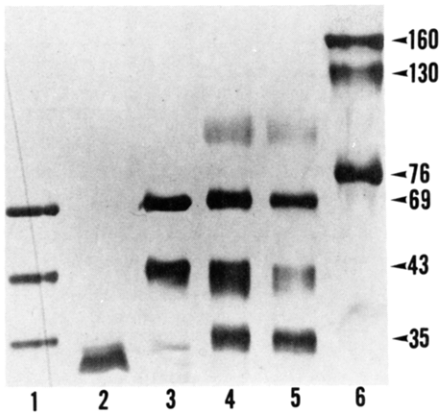

FIGURE 2: SDS–PAGE of $fVIIIa_{IIa}$. Samples from the experiment described in Figure 1 were subjected to SDS–PAGE, followed by silver staining as described under Experimental Procedures. Lane 1, HPLC fraction 20; lane 2, HPLC fraction 13; lanes 3–5 (non-HPLC fractions) fVIII reacted with thrombin for 24 h (lane 3), 4 min (lane 4), or 2 min (lane 5); lane 6, fVIII prior to reaction with thrombin. The markers correspond to apparent molecular masses in kilodaltons.

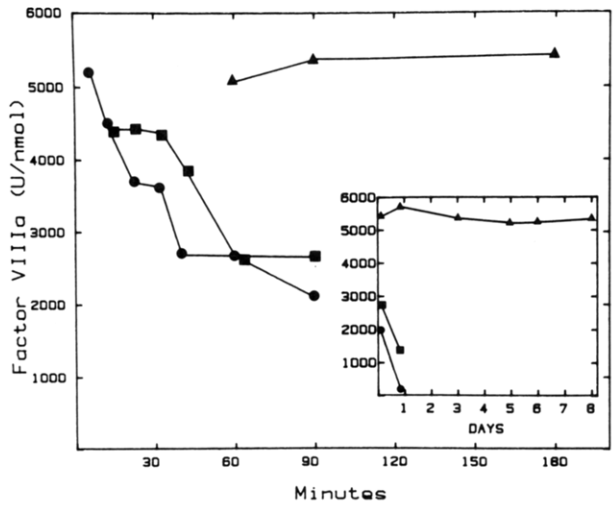

FIGURE 3: Stabilization of $fVIIIa_{IIa}$. Samples corresponding to the experiment described in Figure 1 were assayed at the indicated times for $fVIIIa_{IIa}$ activity as described under Experimental Procedures. Closed circles, non-HPLC sample without PPACK; squares, non-HPLC sample with PPACK added at 7 min; triangles, HPLC fraction 20.

PPACK to the sample at 7 min did not stabilize the activity, which is consistent with the previous observations of the activation of partially purified human fVIII–vWF complex by thrombin (Hultin & Jesty, 1981). Comparison of the proteolytic pattern observed at peak activation to that seen at 1 h showed that the substantial loss of activity during this period did not correspond to an identifiable cleavage (data not shown). After exposure to thrombin for 24 h, proteolysis of $fVIII_{A2}$ to two identifiable poorly stainable fragments with apparent molecular weights less than 20 000 was seen (Figure 2, lane 3). This corresponded to essentially complete loss of $fVIIIa_{IIa}$ activity (Figure 3, inset). PPACK prevented the proteolytic cleavage of $fVIII_{A2}$ (data not shown) but did not completely stabilize $fVIIIa_{IIa}$ activity.

The activation of fVIII by thrombin also was done under identical conditions by using a species of fVIII, $fVIII_{A1\text{-}A2/LC}$, that lacks the entire B domain. The activation/inactivation kinetics and proteolytic pattern were very similar to those described in Figures 2 and 3, indicating that the B domain has little or no effect on the activation or inactivation of fVIII in agreement with similar experiments with human fVIII (Andersson et al., 1986; Fay et al., 1986).

Interestingly, the activity that was eluted from the HPLC column was stable (Figure 3, inset). No loss of activity was detected in any of eight preparations of $fVIIIa_{IIa}$ that were stored at either 4 °C or room temperature for up to 2 months. The activity is reported in units per nanomole to allow direct comparison of the coagulant activity of purified $fVIIIa_{IIa}$ with that of the activated material in the activation mixture. It is evident that there is not a significant loss of specific activity during the HPLC step (Figure 3). The specific activity and yield from the HPLC step of $fVIIIa_{IIa}$ are summarized in Table I. In initial preparations, the solvent for the Mono S step contained Tween 80, a nonionic detergent, in an attempt to prevent loss of protein due to adsorption to column components. Because of the complexity of interpreting hydrodynamic data obtained from systems containing detergents (Tanford & Reynolds, 1976), Tween 80 was omitted from two preparations, including the one described in Figures 1–3. This did not decrease the fractional yield (Table I), and therefore Tween 80 is no longer included in the Mono S step.

Although the mechanism responsible for the stabilization of $fVIIIa_{IIa}$ that occurs during the HPLC step has not been established, several possibilities were excluded. One possibility is that $fVIIIa_{IIa}$ is concentrated by the HPLC step, which drives a reversible association between subunits. Incompletely associated species would have less activity. This possibility is not consistent with the following observation. HPLC fractions containing $fVIIIa_{IIa}$ at concentrations as low as 0.025 mg/mL (0.17 μM) corresponding to the lower limit for accurate absorbance measurements, did not have a lower specific activity than more concentrated fractions (Figure 1). Since the activation mixture contained a higher concentration of fVIII and contained labile activity, this indicates that reversible subunit dissociation of $fVIIIa_{IIa}$ cannot constitute a major mechanism for loss of activity under the conditions used in this experiment.

Another possibility is that $fVIIIa_{IIa}$ emerges from the column in a solvent that stabilizes the active species. FVIII activation by thrombin in this study was done at ionic strength 0.17, pH 7.3, whereas $fVIIIa_{IIa}$ emerged from the column and was stored at ionic strength 0.65, pH 6.0. Therefore, the solvent conditions were tested by diluting $fVIIIa_{IIa}$ into either (a) 0.15 M NaCl/0.02 M Tris-HCl/5 mM $CaCl_2$ at pH 7.4, (b) 0.65 M NaCl/0.02 M Tris-HCl/5 mM $CaCl_2$ at pH 7.4, (c) 0.15 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$ at pH 6.0, or (d) 0.65 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$ at pH 6.0. $FVIIIa_{IIa}$ was stable for at least 24 h in all of these buffers, indicating that its stability is not a function of the particular HPLC elution buffer.

Subunit Structure of $FVIIIa_{IIa}$. $FVIIIa_{IIa}$ appears to consist of a heterodimer since it elutes as a single peak from a monodisperse resin and SDS–PAGE reveals three bands with approximately equal staining intensity. This was confirmed by analytical ultracentrifugation of the $fVIIIa_{IIa}$ preparation. Additionally, these measurements provide information about the shape of the heterotrimer. Velocity sedimentation revealed a single boundary and a sedimentation coefficient ($s^0_{20,w}$) of 7.2 S (Figure 4). Since three fragments of fVIII corresponding to $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ were present, the following combinations of species are possible: (1) $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$, (2) $fVIII_{A1/A3\text{-}C1\text{-}C2}$ and $fVIII_{A2}$, (3) $fVIII_{A2/A3\text{-}C1\text{-}C2}$ and $fVIII_{A1}$, (4) $fVIII_{A3\text{-}C1\text{-}C2}$ and $fVIII_{A1/A2}$, and (5) $fVIII_{A3\text{-}C1\text{-}C2}$, $fVIII_{A1}$, and $FVIII_{A2}$. The finding of a single boundary excludes possibilities 2 and 3. The sedimentation coefficient of $fVIII_{A3\text{-}C1\text{-}C2}$ was measured to be 4.9 S. The

Table II: Equilibrium Sedimentation of $FVIIIa_{IIa}$

		13 000 rpm			15 000 rpm		
prepn	c_o[a] (mg/mL)	M_r(app)	rmsd[b] (A_{280})	base line[c] (A_{280})	M_r(app)	rmsd (A_{280})	base line (A_{280})
1	0.063	147 800	0.0026	0.0026	145 200	0.0026	0.0021
2	0.081	148 700	0.0017	0.0029	148 300	0.0023	0.0021
1	0.12	153 800	0.0029	0.0092	153 200	0.0032	0.0084
2	0.12	157 600	0.0022	0.0055	155 100	0.0025	0.0031
2	0.16	160 700	0.0028	0.0076	160 200	0.0026	0.0076

[a] Loading concentration. [b] Root mean squared deviation of absorbance values and best-fit values. [c] Difference between best-fit base line and base line observed after accelerating the rotor to 52 000 rpm.

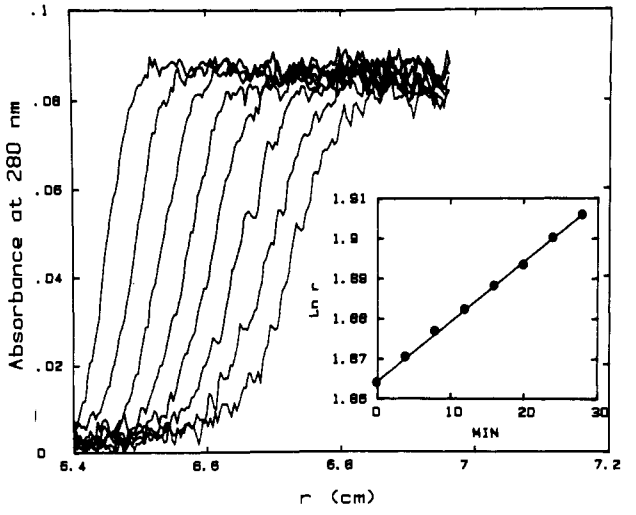

FIGURE 4: Velocity sedimentation of $fVIIIa_{IIa}$. $FVIIIa_{IIa}$, 0.05 mg/mL in 0.65 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$ at pH 6.0, was sedimented at 60 000 rpm in a Beckman Model E analytical ultracentrifuge as described under Experimental Procedures. Shown are the superpositions of eight scans that were obtained at 4-min intervals. The inset shows the plot of the natural logarithm of the equivalent boundary position versus time that was used to calculate the sedimentation coefficient.

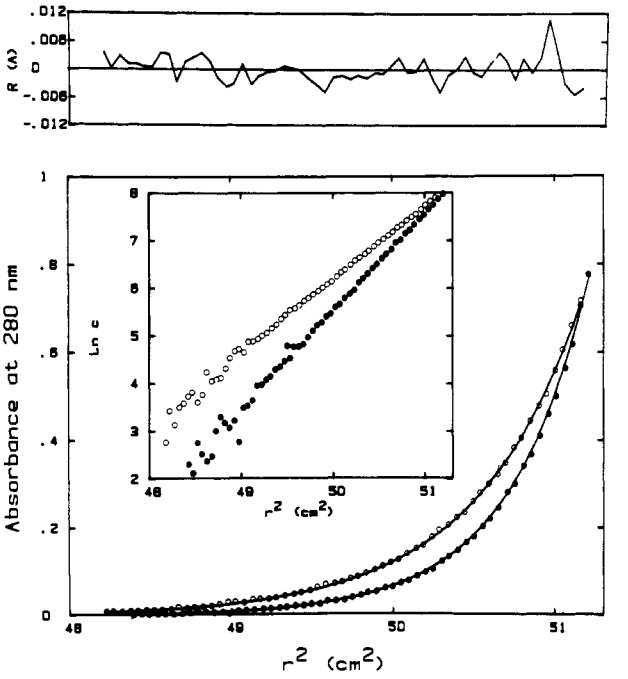

FIGURE 5: Equilibrium sedimentation of $fVIIIa_{IIa}$. $FVIIIa_{IIa}$, 0.12 mg/mL in 0.65 M NaCl/0.01 M His-HCl/5 mM $CaCl_2$ at pH 6.0, underwent analytical ultracentrifugation at 13 000 rpm (open circles) and 15 000 rpm (solid circles) as described under Experimental Procedures. The curves represent the nonlinear least-squares fits to the data. In the upper figure are plotted the residuals corresponding to the data obtained at 13 000 rpm. The inset shows the plots of the natural logarithm of absorbance versus the squared radial position for all values greater than 0.009 A.

absence of an observed boundary moving slower than 7.2 S thus excludes possibilites 4 and 5. Therefore, the velocity sedimentation data are consistent with a single major heterotrimeric species.

The proposed heterotrimeric structure also was supported by equilibrium sedimentation analysis. Figure 5 shows the results of a typical experiment in which data were obtained at two rotor speeds. The absorbance versus squared radial position values were fit to the equation describing a single sedimenting species plus a nonsedimenting base line by using nonlinear least-squares regression (see Experimental Procedures). This allows the calculation of the apparent molecular weight, M_r(app), of the species. Good fits were obtained as indicated by the calculated curves going through the data and the fact that no apparent trend in the residuals is seen as a function of radial position. Sample homogeneity is also apparent from plots of the logarithm of the concentration versus squared radial position, which appeared linear over a 100-fold concentration range (Figure 5, inset). The scatter observed at the lowest squared radial positions is due to the fact that all absorbances greater than 0.009 are plotted. The data in Figure 5 correspond to line 4 in Table II in which the apparent molecular weights corresponding to four different loading concentrations and two preparations of $fVIIIa_{IIa}$ have been calculated at each rotor speed from the effective reduced molecular weights. The M_r(app) values for this sample and all others in Table II change only slightly with rotor speed, which is consistent with sample homogeneity and also indicates that $fVIIIa_{IIa}$ is stable in the centrifuge cell over the 3-day period that was used to collect data. The coefficients of variation for the effective reduced molecular weights calculated from the nonlinear least-squares regression were less that 2% in all cases. The quality of the fits is also indicated by the root mean squared deviations of the data sets, which were less than 0.003 A_{280} in all cases. This is similar to the random error resulting from measurements at a single radial position and indicates that attempts to fit a single data set to more complex schemes would not be fruitful. Additionally, an estimate of low molecular weight material absorbing at 280 nm was obtained by accelerating the rotor to 52 000 rpm, allowing sedimentation for 30 min to obtain an experimental base line, and comparing this value to the base line calculated from the regression analysis. The highest amount of low molecular weight material (0.009 A) was evident from the values in line 3 of Table II. This corresponds to 4.6% of the initial loading concentration when the latter is converted to A_{280} by using the extinction coefficient of $fVIIIa_{IIa}$. Calculation of low molecular weight material ranged from 1.6% to 4.6% for all of the samples and excludes the possibility of a large amount of a nonassociated species.

Although the analysis of data at a single loading concentration is consistent with a single sedimenting species, the apparent molecular weight showed a significant increase

Table III: Hydrodynamic Properties of Porcine $FVIIIa_{IIa}$ and Bovine Factor Va

	$fVIIIa_{IIa}$	factor Va[a]
M_r	150 000–160 000	174 000
$s^0_{20,w}$ (S)	7.2	8.2
$\bar{v}$ (mL/g)	0.73[d]	0.72
f[b] ($\times 10^8$) (g/s)	9.2–9.8	10.0
f/f_{min}[c]	1.39–1.43	1.39
subunits	3	2

[a] Laue et al. (1984). [b] Frictional coefficient. [c] Frictional coefficient ratio. [d] Calculated from human $fVIIIa_{IIa}$ sequence.

ranging from 147 800 to 160 700 at 13 000 rpm and from 145 200 to 160 200 at 15 000 rpm as the loading concentration was increased from 0.062 to 0.16 mg/mL (Table II). In comparison, the summed molecular weight of the porcine $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ bands by SDS–PAGE is 147 000 (Lollar et al., 1985). The summed polypeptide molecular weight deduced from the cDNA of human $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ is 159 000, assuming that these subunits correspond to residues 1–372, 373–740, and 1690–2332 of fVIII, respectively (Vehar et al., 1984; Toole et al., 1984). The possibility that porcine $fVIIIa_{IIa}$ may have a lower molecular weight than human $fVIIIa_{IIa}$ was made less likely by the fact that the apparent molecular weights of the proposed heterotrimer from SDS–PAGE analysis of thrombin-cleaved porcine and recombinant human fVIII were equal (data not shown). The M_r(app) values clearly establish the major species in the preparation as the heterotrimer. However, variation in M_r(app) with loading concentration also indicates the presence of additional minor species and is characteristic of an associating system. Whether this represents weak self-association of the $fVIIIa_{IIa}$ heterotrimer to form a hexamer and/or incomplete association of the three subunits cannot be determined from the data. In contrast, solution nonideality is very unlikely given the small loading concentrations and the fact that nonideality usually causes the apparent molecular weight of a protein to decrease as the loading concentration is increased (Tanford, 1961). Additionally, macromolecular heterogeneity due to noninteracting species does not result in a change in apparent molecular weight as a function of loading concentration (Yphantis, 1964).

Calculated Hydrodynamic Parameters of $FVIIIa_{IIa}$. The frictional coefficient and frictional coefficient ratio of $fVIIIa_{IIa}$ were calculated from its molecular weight, sedimentation coefficient, and partial specific volume. A summary of the hydrodynamic data for $fVIIIa_{IIa}$ is given in Table III. Values are reported for a molecular weight range of 150 000–160 000. The frictional coefficient ratio gives a measure of the departure of the molecular shape from a sphere since an anhydrous sphere gives a value of 1. A compilation of frictional coefficient ratios for several symmetrical proteins with known crystallographic structure ranging from nearly spherical, monomeric proteins to four-subunit tetrahedral proteins listed values ranging from 1.05 to 1.34 (Creighton, 1983). Thus, in comparison, $fVIIIa_{IIa}$ appears to be a moderately asymmetrical molecule. Also shown in Table III are the corresponding hydrodynamic values for bovine thrombin-activated factor V (factor Va) (Laue et al., 1984). The similarity between the frictional coefficient ratios indicates that $fVIIIa_{IIa}$ and factor Va have a similar quaternary structure despite having an unequal number of subunits.

Discussion

FVIII must be proteolytically activated to function as a cofactor in the activation of factor X by factor IXa. In this study of the activation of fVIII by thrombin, we find that it is possible to obtain a stable preparation of porcine $fVIIIa_{IIa}$ containing as the major species a heterotrimer composed of the subunits $fVIII_{A1}$, $fVIII_{A2}$, $fVIII_{A3\text{-}C1\text{-}C2}$. Although it has been reported that the activation of highly purified fVIII by thrombin yields a stable product (Vehar & Davie, 1980; Eaton et al., 1986a, 1987), the stability has not been reported beyond 2 h, and this product has not been isolated. Additionally, it has not been shown that the fVIII fragments have been cleaved completely during the period of apparent stability, which raises the possibility of a steady-state level of activation and inactivation. In this study, a preparation of $fVIIIa_{IIa}$ has been obtained in an equilibrium state that is free of thrombin and fVIII activation peptides.

Several studies using either highly purified fVIII or fVIII containing vWF and other contaminants have found that $fVIIIa_{IIa}$ is unstable (Cooper et al., 1975; Hultin & Jesty, 1981; Fulcher et al., 1983; Lollar et al., 1984, 1985; Lollar & Fass, 1984; Rotblat et al., 1985; Andersson et al., 1986; Fay et al., 1986; Fay, 1987). Studies using highly purified porcine fVIII have addressed this phenomenon. Fass et al. (1982) originally proposed that porcine fVIII is isolated from plasma as a set of heterodimers defined as 166/76- and 130/76-kDa species by their apparent SDS–PAGE molecular masses. The common 76-kDa fragment is designated $fVIII_{LC}$ with the other fragments being called heavy-chain species. This hypothesis is now supported by several lines of evidence from work on both human and porcine fVIII (Vehar et al., 1984; Toole et al., 1984; Fay et al., 1986; Andersson et al., 1986; Nordfang & Ezban, 1988). Subsequently, an 82-kDa species, corresponding to $fVIII_{A1\text{-}A2}$, with an identical amino-terminal sequence to the 166- and 130-kDa species was identified in some preparations (Toole et al., 1984). The 166/76-, 130/76-, and 82/76-kDa species arise from proteolysis in the middle or B domain of the parent single-chain fVIII molecule. The addition of thrombin to a mixture of these species results in cleavage of $fVIII_{LC}$ at a position equivalent to residue 1689 in human fVIII to form $fVIII_{A3\text{-}C1\text{-}C2}$ and cleavages of the heavy-chain species to form $fVIII_{A1}$ and $fVIII_{A2}$. N-Terminal sequencing of human and porcine $fVIII_{A2}$ has identified one cleavage site as arginine-372 between the A1 and A2 domains (Vehar et al., 1984; Toole et al., 1984; Eaton et al., 1986). The C-terminal residue of $fVIII_{A2}$ has been tentatively identified as arginine-740 because a fVIII activation peptide has been isolated with an N-terminal sequence starting at position 741 (Toole et al., 1984).

Whether cleavage of $fVIII_{A1\text{-}A2}$ between the A1 and A2 domains at position 372 is necessary for activation of fVIII has been controversial. The activation of porcine fVIII by a molar excess of thrombin has been evaluated with the following results: (1) In the absence of thrombin the fVIII preparation has little or no ability to support the activation of factor X by factor IXa. (2) $FVIIIa_{IIa}$ is unstable with a half-life of 5–10 min. (3) The loss of activity is not associated with further identifiable proteolytic cleavages by SDS–PAGE. (4) The addition of factor IXa and phospholipid vesicles to the activation mixture increases the half-life of activated fVIII to approximately 1 h without altering the proteolytic cleavage pattern (Lollar & Fass, 1984; Lollar et al., 1984). Additionally, the rate of activation of fVIII by thrombin depends on the concentration of thrombin while the rate of inactivation does not (Lollar et al., 1985). On the basis of these results, it appears that the activation of fVIII involves cleavages at positions 372 and/or 1689 while inactivation is by a nonproteolytic mechanism.

Some studies with human fVIII derived from plasma or recombinant DNA also have observed that the activation of fVIII by thrombin correlates with the appearance of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ (Eaton et al., 1986a, 1987). In contrast, it has been proposed that $fVIIIa_{IIa}$ consists of a $fVIII_{A1\text{-}A2/LC}$ or $fVIII_{A1\text{-}A2/A3\text{-}C1\text{-}C2}$ heterodimer on the basis of the correlation of observed proteolytic cleavages of human fVIII by thrombin with the kinetics of activation of fVIII (Fulcher et al., 1983; Rotblat et al., 1985). The cleavage of $fVIII_{A1\text{-}A2}$ at position 372 was proposed as a mechanism for the inactivation of $fVIIIa_{IIa}$. However, the human $fVIII_{A1\text{-}A2/LC}$ heterodimer has been subsequently isolated and found to require proteolytic activation by thrombin to develop full activity in coagulation assays (Andersson et al., 1986; Fay et al., 1986). These observations are confirmed with the equivalent porcine heterodimer in this study. This apparently excludes the possibility that $fVIII_{A1\text{-}A2/LC}$ constitutes a possible subunit structure of $fVIIIa_{IIa}$. This interpretation is consistent with observations resulting from site-directed mutagenesis of the fVIII cDNA and comparison of recombinant $fVIIIa_{IIa}$ activity with the proteolytic pattern produced by thrombin (Pittman & Kaufman, 1988). Modification of arginine to isoleucine residues at either position 372 or position 1689 resulted in production of fVIII species that could not be activated by thrombin, whereas a similar modification at position 740 resulted in an activatable species of fVIII.

The functional significance of the cleavage at position 372 or position 1689 of the $fVIII_{A1\text{-}A2/LC}$ heterodimer has not been resolved. At this point, the distinction between the activation of fVIII and that of the fVIII–vWF complex must be made. There is general agreement that the binding of fVIII to vWF is mediated at least in part by $fVIII_{LC}$ (Hamer et al., 1987; Foster et al., 1988; Lollar et al., 1988b). It has been reported that $fVIII_{A2}$ also binds vWF and remains bound after cleavage of fVIII by thrombin while $fVIII_{LC}$ is released (Hamer et al., 1987). However, we have observed by analytical velocity sedimentation that (1) the 166/76-kDa fVIII heterodimer and $fVIII_{LC}$ bind vWF with equivalent stoichiometry; (2) 166-, 130-, and 82-kDa fragments do not bind vWF; (3) $fVIII_{A3\text{-}C1\text{-}C2}$ does not bind vWF; and (4) cleavage of the fVIII–vWF complex is associated with the dissociation of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ from vWF (Lollar & Parker, 1987; Lollar et al., 1988b). This suggests that the binding of fVIII to vWF is mediated solely by $fVIII_{LC}$ and that cleavage at position 1689 releases fVIII from vWF. This is consistent with the observation that antibodies to a synthetic peptide corresponding to fVIII residues 1670–1684 block the binding of fVIII to vWF (Foster et al., 1988). Therefore, it is conceivable that, in the absence of vWF, only cleavage at position 372 is necessary for the development of intrinsic procoagulant activity.

In the present study, a molar excess of fVIII was activated by thrombin. In the reaction mixture, the coagulant activity generated by thrombin was unstable and could not be stabilized by the addition of an irreversible active-site-directed inhibitor of thrombin. Most of the activity was lost by 1 h (Figure 3) without any apparent proteolysis of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$. After 1 day, there was essentially no activity remaining in the sample without PPACK and there was proteolysis of $fVIII_{A2}$ (Figure 2). The presence of PPACK prevented this proteolysis but not the continued loss of activity. Thus, it appears that most of the loss of coagulant activity can be accounted for by a nonproteolytic mechanism. The decay of activity in Figure 1 was slower than that seen in an earlier study with porcine fVIII in which a lower concentration of fVIII was used (Lollar et al., 1984). This suggests that an additional nonproteolytic mechanism may be involved at concentrations of $fVIIIa_{IIa}$ lower than those used in this study (e.g., subunit dissociation).

HPLC of the reaction mixture after peak activation by thrombin resulted in the isolation of a stable product (Figures 1 and 3). This does not appear to be due to a change in solvent conditions since dilution of the stable $fVIIIa_{IIa}$ into a variety of buffers did not destabilize the activity. Additionally, subunit dissociation cannot account for the effect since concentrations of stable $fVIIIa_{IIa}$ as low as those in the reaction mixture were obtained. It appears that the most likely explanation for the stabilizing effect of the chromatographic step is the removal of an as yet unidentified inhibitor of $fVIIIa_{IIa}$. One possible inhibitor is thrombin. Since most of the loss of activity was not associated with an identifiable proteolytic cleavage and did not depend on the active site of thrombin, we cannot exclude that this inhibition is due to a separate enzymatic function of thrombin.

Another possibility is the inhibition of $fVIIIa_{IIa}$ by a fragment of the fVIII molecule itself. Since the activation of human $fVIII_{A1\text{-}A2/LC}$, which lacks the entire B domain, leads to an unstable product, this domain does not appear to be involved (Andersson et al., 1986; Fay et al., 1986). We obtained similar results in this study with porcine $fVIII_{A1\text{-}A2/LC}$, confirming this observation. Fragments that cannot be excluded are the 41-residue N-terminal fragment of $fVIII_{LC}$ and C-terminal fragments of $fVIII_{A2}$ and $fVIII_{A3\text{-}C1\text{-}C2}$.

The subunit structure of the isolated, stable $fVIIIa_{IIa}$ appears to be $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ by the presence of three bands after SDS–PAGE that stain with equal intensity (Figure 2). Sedimentation data confirm that the major species in the preparation has this subunit structure. The finding of a single boundary during velocity sedimentation is not consistent with the presence of a large amount of a nonassociated fragment (Figure 4). The M_r(app) values obtained from equilibrium sedimentation also are consistent with the proposed structure (see Results). Additionally, the increase in M_r(app) with loading concentration indicates the presence of minor species due to an associating system (see Results). Whether this is due to self-association of the heterotrimer, incomplete association of the three subunits to form the heterotrimer, or both is not clear.

In contrast to porcine $fVIIIa_{IIa}$, bovine factor Va is a heterodimer (Esmon, 1979). The subunit structure of bovine factor Va has been studied in detail by analytical ultracentrifugation (Laue et al., 1984). Factor Va consists of a M_r 92 300 heavy chain and a M_r 82 500 light chain. The two chains are homologous to $fVIII_{A1\text{-}A2}$ and $fVIII_{LC}$, respectively (Fass et al., 1985; Kane & Davie, 1986; Jenny et al., 1987). Association of these two chains in the ultracentrifuge was too tight to allow an accurate estimate of the association constant ($K > 2.7 \times 10^8$ M^{-1}). Additionally, there was clear evidence of a weak self-association of the heterodimer to form at least a species consisting of two heterodimers ($K = 3000$ M^{-1}) with perhaps higher order associations occurring. In the absence of equilibrium sedimentation data on isolated $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$, the nature of the minor species in the $fVIIIa_{IIa}$ preparation must remain speculative.

The difference in the number of subunits between factor Va and $fVIIIa_{IIa}$ is due to the absence of a thrombin cleavage site in factor Va between the A1 and A2 domains. However, the frictional coefficient ratios of $fVIIIa_{IIa}$ and factor Va are nearly identical. This suggests that the quaternary structures of the two proteins are similar or equivalently that the generation of an additional subunit in $fVIIIa_{IIa}$ does not result

in a displacement of the A1 or A2 domains relative to the light chain.

It has been proposed that human $fVIIIa_{IIa}$ is a $fVIII_{A1/A3\text{-}C1\text{-}C2}$ heterodimer (Fay, 1987; Hamer et al., 1987) although the finding of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ in a single fraction following gel permeation chromatography of $fVIIIa_{IIa}$ does not support these studies (Eaton et al., 1987). Hamer et al. (1987) studied the binding of ^{125}I-fVIII to vWF immobilized to plastic. The addition of thrombin to the solid-phase ^{125}I-fVIII–vWF complex resulted in the release of $fVIII_{A1}$ and $fVIII_{A3\text{-}C1\text{-}C2}$ into the supernatant. The activity of the bound or released material was not measured. From this experiment and the observation that ^{125}I-$fVIII_{LC}$ binds vWF in this system, it was proposed that (1) vWF has binding sites for both $fVIII_{LC}$ and $fVIII_{A2}$; (2) the activation of the fVIII–vWF complex by thrombin results in the release of the active $fVIII_{A1/A3\text{-}C1\text{-}C2}$ heterodimer; and (3) $fVIII_{A2}$ remains bound to vWF.

Fay studied the activation of fVIII by thrombin by using activity measurements and by SDS–PAGE of fVIII fragments following HPLC of the activated reaction mixture. In the absence of a chromatography step, the proteolytic pattern and the kinetics of activation and inactivation occurred in a manner very similar to that observed in this study. Gel filtration (Superose 12) or anion-exchange (Mono Q) chromatography of the reaction mixture resulted in the isolation of a product with unstable activity. SDS–PAGE of the fractions that contained activity showed bands corresponding to the $fVIII_{A1}$ and $fVIII_{A3\text{-}C1\text{-}C2}$ subunits with only a small amount of material corresponding to $fVIII_{A2}$. Hydrodynamic measurements using analytical gel filtration and sucrose density gradient velocity sedimentation were consistent with $fVIII_{A1}$ and $fVIII_{A3\text{-}C1\text{-}C2}$ subunits being associated.

One hypothesis that explains the discrepancy between these studies and ours regarding the subunit structure of $fVIIIa_{IIa}$ is that $fVIII_{A2}$ has a strong tendency to bind certain surfaces nonspecifically. This would completely explain the findings of the Hamer study. The correlation between activity and presence of the $fVIII_{A1}$ and $fVIII_{A3\text{-}C1\text{-}C2}$ fragments in the Fay study then could be due to one of several possibilities. First, there is possibly a small amount of $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ in the preparation that accounts for all of the activity. Second, the $fVIII_{A1/A3\text{-}C1\text{-}C2}$ heterodimer could have activity that is less than or equal to that of $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$, but the lack of the $fVIII_{A2}$ subunit results in an unstable species. Third, the $fVIII_{A1/A3\text{-}C1\text{-}C2}$ heterodimer may be fully active, and the observed loss of activity is independent of subunit structure.

The labile nature of $fVIIIa_{IIa}$ thus remains poorly understood. Hopefully, the ability to obtain a preparation of stable $fVIIIa_{IIa}$ will be important in designing experiments to determine the mechanisms of the activation and inactivation of fVIII.

Acknowledgments

We thank Dr. David N. Fass for his gift of the cell line producing the monoclonal antibody used to isolate fVIII and Dr. Dan Eaton for his gift of recombinant human fVIII. We acknowledge the helpful discussions of David C. Hill-Eubanks.

Registry No. fVIII, 113189-02-9; $fVIII_{IIa}$, 72175-66-7; thrombin, 9002-04-4.

References

Andersson, L. O., Forsman, N., Huang, K., Larsen, K., Lundin, A., Pavlu, B., Sandberg, J., Sewerin, K., & Smart, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2979–2983.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 204–242, McGraw-Hill, New York.

Cantor, C. R., & Schimmel, P. R. (1980a) *Biophysical Chemistry. Part II. Techniques for the Study of Biological Structure and Function*, pp 591–642, Freeman, San Francisco.

Cantor, C. R., & Schimmel, P. R. (1980b) *Biophysical Chemistry. Part II. Techniques for the Study of Biological Structure and Function*, pp 539–590, Freeman, San Francisco.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino-Acids and Peptides as Dipolar Ions*, p 370, Reinhold, New York.

Cooper, H. A., Reisner, F. F., Hall, M., & Wagner, R. H. (1975) *J. Clin. Invest. 56*, 751–760.

Creighton, T. E. (1983) *Proteins. Structures and Molecular Properties*, pp 265–334, Freeman, New York.

Eaton, D., Rodriguez, H., & Vehar, G. A. (1986a) *Biochemistry 25*, 505–512.

Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vehar, G. A., & Gorman, C. (1986b) *Biochemistry 25*, 8343–8347.

Eaton, D. L., Hass, P. E., Riddle, L., Mather, J., Wiebe, M., Gregory, T., & Vehar, G. A. (1987) *J. Biol. Chem. 262*, 3285–3290.

Esmon, C. T. (1979) *J. Biol. Chem. 254*, 964–973.

Fass, D. N., Knutson, G. J., & Katzmann, J. A. (1982) *Blood 59*, 594–600.

Fass, D. N., Hewick, R. M., Knutson, G. J., Nesheim, M. E., & Mann, K. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1688–1691.

Fay, P. J. (1987) *Biochim. Biophys. Acta 952*, 181–190.

Fay, P. J., Anderson, M. T., Chavin, S. I., & Marder, V. J. (1986) *Biochim. Biophys. Acta 871*, 268–278.

Foster, P. A., Fulcher, C. A., Houghten, R. A., & Zimmerman, T. S. (1988) *J. Biol. Chem. 263*, 5230–5234.

Fulcher, C. A., & Zimmerman, T. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1648–1652.

Fulcher, C. A., Roberts, J. R., & Zimmerman, T. S. (1983) *Blood 61*, 807–811.

Gitschier, J., Wood, W. I., Goralka, T. M., Wion, K. L., Chen, E. Y., Eaton, D. H., Vehar, G. A., Capon, D. J., & Lawn, R. M. (1984) *Nature (London) 312*, 326–330.

Hamer, R. J., Koedam, J. A., Beeser-Visser, N. H., & Sixma, J. J. (1987) *Eur. J. Biochem. 167*, 253–259.

Hultin, M. B., & Jesty, J. (1981) *Blood 57*, 476–482.

Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., & Mann, K. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4846–4850.

Kane, W. H., & Davie, E. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6800–6804.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laue, T. M., Johnson, A. E., Esmon, C. T., & Yphantis, D. A. (1984) *Biochemistry 23*, 1339–1348.

Lollar, P., & Fass, D. N. (1984) *Arch. Biochem. Biophys. 233*, 438–446.

Lollar, P., & Parker, C. G. (1987) *J. Biol. Chem. 262*, 17572–17576.

Lollar, P., Knutson, G. J., & Fass, D. N. (1984) *Blood 63*, 1303–1308.

Lollar, P., Knutson, G. J., & Fass, D. N. (1985) *Biochemistry 24*, 8056–8064.

Lollar, P., Parker, C. G., Kajenski, P. J., Litwiller, R. D., & Fass, D. N. (1987) *Biochemistry 26*, 7627–7636.

Lollar, P., Parker, C. G., & Tracy, R. P. (1988a) *Blood 71*, 137-143.
Lollar, P., Hill-Eubanks, D. C., & Parker, C. G. (1988b) *J. Biol. Chem. 263*, 10451-10455.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307-310.
Nordfang, O., & Ezban, M. (1988) *J. Biol. Chem. 263*, 1115-1118.
Pittman, D. D., & Kaufman, R. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2429-2433.
Rotblat, F., O'Brien, D. P., O'Brien, F. J., Goodall, A. H., & Tuddenham, E. G. (1985) *Biochemistry 24*, 4294-4300.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 180-274, Wiley, New York.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133-170.
Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C., Orr, E. C., Amphlett, G. W., Foster, W. B., Coe, M. L., Knutson, G. J., Fass, D. N., & Hewick, R. M. (1984) *Nature (London) 312*, 342-347.
van Iersel, J., Jzn, J. F., & Duine, J. A. (1985) *Anal. Biochem. 151*, 196-204.
Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401-410.
Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M., & Capon, D. J. (1984) *Nature (London) 312*, 337-342.
Wood, W. I., Capon, D. J., Simonsen, C. C., Eaton, D. L., Gitschier, J., Keyt, B., Seeburg, P. H., Smith, D. H., Hollingshead, P., Wion, K. L., Delwart, E., Tuddenham, E. G. D., Vehar, G. A., & Lawn, R. M. (1984) *Nature (London) 312*, 330-337.
Yphantis, D. A. (1964) *Biochemistry 3*, 297-317.

Primary Structure of a Protein C Activator from *Agkistrodon contortrix contortrix* Venom†

Brad A. McMullen,‡ Kazuo Fujikawa,‡ and Walter Kisiel*,§
Department of Biochemistry, University of Washington, Seattle, Washington 98195, and Department of Pathology, School of Medicine, University of New Mexico, Albuquerque, New Mexico 87131
Received August 4, 1988; Revised Manuscript Received September 7, 1988

ABSTRACT: The amino acid sequence of a protease, protein C activator, from *Agkistrodon contortrix contortrix* venom was determined. Peptide fragments obtained by chemical or enzymatic cleavage of the S-carboxymethylated protein were purified by gel filtration and reverse-phase high-performance liquid chromatography. The present study demonstrates that protein C activator from *A. contortrix contortrix* venom is a trypsin-type serine protease that is composed of 231 residues with a molecular weight of 25 095 for the polypeptide portion of the molecule. By analogy to the mammalian serine proteases, the catalytic triad in venom protein C activator consists of His-40, Asp-85, and Ser-177. The protein also contains three N-linked glycosylation sites at Asn-21, Asn-78, and Asn-129. The amino acid sequence of protein C activator exhibits a high degree of sequence identity with other snake venom proteases: 73% with batroxobin, 68% with flavoxobin, and 55% with Russell's viper venom factor V activator.

Protein C is a two-chain vitamin K dependent protein that circulates in mammalian blood as a precursor to a serine protease (Stenflo, 1976; Esmon et al., 1976; Kisiel et al., 1976; Kisiel, 1979). Human protein C is activated in vivo by the proteolytic release of a dodecapeptide from the amino-terminal end of its heavy chain by a complex of thrombin and thrombomodulin on the endothelial cell surface (Esmon & Owen, 1981). Activated protein C, in contrast to the activated vitamin K dependent coagulant factors, exhibits strong anticoagulant activity through its ability to degrade rapidly and specifically factors Va and VIIIa (Kisiel et al., 1977; Suzuki et al., 1983; Eaton et al., 1986).

Protein C is also activated in vitro by other nonphysiological activators, such as trypsin (Esmon et al., 1976; Kisiel et al., 1976) and the factor X activator from Russell's viper venom (Kisiel et al., 1976). Recently, the crude venom of the Southern Copperhead snake (*Agkistrodon contortrix contortrix*) has been shown to activate protein C rapidly (Stocker et al., 1986). A protease, protein C activator, has now been purified to homogeneity from Southern Copperhead venom in a number of laboratories (Stocker et al., 1986; Kisiel et al., 1987a; Exner & Vaasjoki, 1988; Orthner et al., 1988), and one of these preparations is commercially available under the trade name Protac. Protac has been used successfully in the development of clinically useful, sensitive functional assays for both protein C (Martinoli & Stocker, 1986; Frances & Seyfert, 1987; McCall et al., 1987) and protein S, a cofactor for the expression of activated protein C anticoagulant activity (Suzuki & Nishioka, 1988).

The molecular properties of the venom protein C activator, designated in this paper ACC-C,[1] have been described in detail

† This work was supported in part by grants from the National Institutes of Health (HL 16919 and HL 35246) and by Blood Systems, Inc.
* Address correspondence to this author.
‡ University of Washington.
§ University of New Mexico.

[1] Abbreviations: ACC-C, a protease from *Agkistrodon contortrix contortrix* venom that activates protein C; BNPS-skatole, 2-(2′-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; CM, carboxymethyl; CNBr, cyanogen bromide; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; kDa, kilodaltons; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; PMSF, phenylmethanesulfonyl fluoride; aPMSF, *p*-amidinophenylmethanesulfonyl fluoride; PPACK, D-Phe-Pro-Arg chloromethyl ketone; PTH, phenylthiohydantoin; RVV-V, a protease from Russell's viper venom that activates factor V; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

0006-2960/89/0428-0674$01.50/0