Thrombin-activated porcine factor VIII (fVIIIa) is a stable, active, 160-kDa heterotrimer at concentrations exceeding $2 \times 10^{-7}$ M in 0.7 M NaCl, 0.01 M histidine Cl, 5 mM CaCl$_2$, pH 6.0, at 4 °C or 20 °C. Two of the subunits, fVIII$\alpha_1$, and fVIII$\alpha_2$, are derived from the heavy chain of the plasma-derived, heterodimeric fVIII precursor. The third subunit, fVIII$\alpha_3$-C1-C2, is derived from the fVIII light chain. We now find that fVIIIa undergoes a sharp decline in coagulant activity between pH 7 and 8. At pH 7.5, the activity of fVIIIa at $3 \times 10^{-7}$ M decays within a few hours to a stable level that is approximately 70% of the value at pH 6.0, whereas at pH 8.0, greater than 99% of the activity is lost. The activity cannot be restored by readjusting the pH to 6.0. The loss of activity at pH 8.0 coincides with dissociation of the fVIII$\alpha_3$ subunit since an inactive fVIII$\alpha_3$-C1-C2 heterodimer can be isolated from fVIIIa by pH-dependent subunit dissociation. The free fVIII$\alpha_3$, fragment appears to be poorly soluble which may explain the irreversible loss of activity. Analytical velocity sedimentation of the pH-inactivated fVIIIa preparation also is consistent with dissociation and precipitation of the fVIII$\alpha_2$ fragment. We propose that denaturation of fVIIIa by pH-dependent subunit dissociation may provide a major mechanism of inactivation of fVIIIa under physiologic conditions.

The proteolytic activation of factor X along the intrinsic pathway of blood coagulation is associated with a single bond cleavage that is catalyzed by factor IXa (1). The reaction is markedly accelerated in the presence of activated factor VIII (fVIIIa), calcium, and a phospholipid membrane surface (2). The mechanism by which fVIIIa participates as a cofactor to increase the catalytic efficiency of factor IXa is unknown.

Recent studies using highly purified fVIII have been directed toward an understanding of the molecular properties of fVIII. fVIII is synthesized as a single chain protein containing internal sequence homology and a domainal sequence A1-A2-B-A3-C1-C2 (3, 4). However, fVIII is isolated from plasma as a set of heterodimers as a result of variable proteolysis in the middle or B domain (5). Three heterodimers in which the heavy chain size is variable and the light chain is constant are commonly isolated from porcine plasma. The heterodimers are pro-cofactors that require proteolytic activation by thrombin (factor IXa) or factor Xa. Thrombin catalyzes cleavages of fVIII heavy chain species at position 462 (between the A1 and A2 domains) and at position 472 (between the A2 and B domains) and the fVIII light chain at position 1689 (6). Although cleavage of fVIII by thrombin is associated with the development of procoagulant or cofactor activity, it has been widely observed that fVIIIa is not stable (7-16). It has been proposed that thrombin activates fVIIIa through further proteolytic cleavages (0, 12). However, the spontaneous loss of fVIIIa activity is not correlated with proteolytic cleavages that can be identified by gel electrophoresis (10). Moreover, we recently found that fVIIIa isolated by cation exchange chromatography at pH 6 is stable when stored at concentrations greater than 0.025 mg/ml (2 x 10^{-4} M) at either 4 or 20 °C (16). Analysis of this preparation revealed that fVIIIa is a heterotrimer consisting of a subunit derived from the light chain and subunits from the A1 and A2 domains of the heavy chain. This finding demonstrates that heavy chain cleavage between the A1 and A2 domains by thrombin activates fVIII and provides the impetus to search for nonproteolytic mechanisms to explain the inactivation of fVIIIa that has been observed. In the present study, we have found that fVIIIa undergoes a marked loss of activity between pH 7 and 8, and that this process is accompanied by subunit dissociation.

**EXPERIMENTAL PROCEDURES**

All procedures were carried out at room temperature unless indicated otherwise.

**Materials—** L-Histidine was purchased from Sigma. Mes and Heps were from BDH Chemicals Ltd. (Poole, England). Activated partial thromboplastin reagent was purchased from General Diagnostics. fVIII-deficient plasma was purchased from George King Biomedical. Mono S and Mono Q (HR 5/5) chromatography matrices were purchased from Pharmacia LKB Biotechnology Inc. The W3-3 hybridoma cell line producing mouse monoclonal anti-porcine fVIII was generously provided by Dr. D. N. Faas, Mayo Clinic/Foundation. Porcine fVIII concentrate was purchased from Porton Products.

**Isolation of Proteins—** fVIII was isolated by using monoclonal anti-fVIII immunofinity chromatography and Mono Q HPLC as previously reported (16). fVIIIa was prepared by activating fVIII with thrombin followed by Mono S HPLC as described (16) except that in some experiments 10 mM Mes was substituted for 10 mM histidine in the column buffers. The specific activity of the fVIIIa preparations ranged from 120,000 to 170,000 units/mg in a two-stage coagulation
assay. fVIIIa was stored in the Mono S column elution buffer at 4° C. The extinction coefficient (A_{280}) at 380 nm and molecular mass of fVIIIa used in the calculations were 1.6 and 160 kDa, respectively (16).

**Assays**—fVIII and fVIIIa were measured by one stage and two stage assays, respectively, in an activated partial thromboplastin time assay using human fVIII-deficient plasma as a substrate as described previously (6). One unit of porcine fVIII was defined as the amount of fVIII in normal citrated porcine plasma.

**Velocity Sedimentation Analysis**—Experiments were done by using a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner as described previously (16). Runs were made with the temperature controller off which resulted in a constant temperature between 20 and 22° C. Measurements were made on 0.25-ml samples in an An-D rotor at 60,000 rpm. Scans were obtained at 4-min intervals. Sedimentation coefficients were determined by estimating the boundary position as the midpoint between meniscus and the plateau absorbance and were corrected for solvent density, viscosity, and temperature (17).

**Electrophoresis**—SDS-PAGE was done using the buffer system of Laemmli (18) under conditions described previously (19) followed by silver staining of the proteins (20). Each lane contained approximately 500–1000 ng.

**RESULTS**

**pH-induced Inactivation of fVIIIa**—fVIIIa was isolated by Mono S chromatography at pH 6.0 and stored at 4° C. The coagulant activity of the preparation was stable for at least several weeks in the elution buffer. Since the activation of fVIII by thrombin at pH 7.4 is associated with a subsequent considerable decline in activity (16), we examined the hypothesis that the stabilization of fVIIIa during its purification on Mono S is at least partly pH-dependent. Fig. 1 shows that fVIIIa activity at 3 × 10^{-7} M is stable between pH 5 and 6, decreases as the pH is raised to 7.5, and is essentially abolished by raising the pH to 8.0. Most of the loss of activity is observed within the first 2 h; activity at 72 h was within 10% of the 6-h value in all cases (data not shown). The inset shows the specific activity at 6 h plotted as a function of pH and demonstrates the marked decline between pH 7 and 8.

The pH-induced, time-dependent loss of fVIIIa activity shown in Fig. 1 could not completely reversed by adjusting the solution back to pH 6.0. In the control reaction, NaOH and HCl were added simultaneously resulting in no change in activity. In Fig. 2, the pH of a solution fVIIIa was increased for 1 h and then readjusted to 6.0. The loss of activity was slightly reversed but not completely. In a control reaction in which the pH was maintained at 8.0, the specific activity continued to decrease. In a separate control reaction, NaOH and HCl were added simultaneously, resulting in no loss of activity. This control indicates that the loss of activity is not due to a contaminant in the reagents used to adjust the pH. Since the activity of fVIIIa is not restored by simply readjusting the pH back to 6.0, the pH-induced inactivation of fVIIIa includes an irreversible step.

**Subunit Dissociation of fVIIIa**—Subunit dissociation of fVIIIa, as a function of pH—fVIIIa at pH 6.0 is a heterotrimer (16). To test whether the heterotrimeric subunit structure of fVIIIa is altered during pH-induced inactivation, the solution was raised to pH 8.0 for either 30 min or 2 h, followed by readjusting the pH to 6.0 and rechromatography on Mono S. After 30 min, only 9% of the activity remained, in agreement with the results shown in Fig. 1. Fig. 3 shows the chromatogram obtained from the 30-min sample. Small amounts of material absorbing at 280 nm are seen in the nonbinding fractions followed by a single major peak emerging from the gradient in 0.76 M NaCl. Column fractions were assayed within 20 min after collection, but there was no detectable activity in either the nonbinding or peak material. Approximately 50% of the protein applied to the column was recovered. After 2 h at pH 8.0, greater than 96% of the activity had disappeared. The resulting Mono S chromatogram gave results similar to those shown in Fig. 3 (data not shown).

SDS-PAGE analysis of the protein fractionated by Mono S after the 30-min and 2-h inactivation reactions is shown in Fig. 4. The material associated with the gradient peak in Fig. 3 mainly contained the thrombin-cleaved light chain (fVIII{sub}A3-C2) and the heavy chain-derived fragment fVIII{sub}A2, with only trace amounts of fVIII{sub}A2, indicating that the major species associated with the peak consists of an inactive heterodimer that lacks the fVIII{sub}A2 fragment. The fVIII{sub}A2 fragment was difficult to recover from the column. After the

![Fig. 1. pH-induced inactivation of fVIIIa.](http://www.jbc.org/)

![Fig. 2. Irreversible inactivation of fVIIIa.](http://www.jbc.org/)

![Fig. 3. SDS-PAGE analysis of the protein fractionated by Mono S.](http://www.jbc.org/)
A solution (0.75 ml) containing FVIIIa, 0.12 mg/ml (8 \times 10^{-6} \text{ M}) in pH 6.0, and applied to a Mono S column equilibrated in 0.1 M NaCl, 5 mM Mes, 5 mM CaCl\(_2\), pH 6.0. After washing the column with 4-5 ml of the equilibration buffer, bound protein was eluted with a 0.1-1.0 M NaCl gradient in the same buffer. One milliliter fractions were collected. The arrow indicates the start of elution. The difficulty recovering the FVIIIA\(_2\) fragment was identified, but only in trace amounts (Fig. 4, lane 2) and could not be identified in other column fractions. The difficulty recovering the FVIIIA\(_2\) fragment indicates that it is either poorly soluble and/or adsorbs irreversibly to the column matrix.

The nonbinding fractions following the 30-min pH 8.0 exposure also contained the FVIIIA\(_1\) fragment, but not FVIIIA\(_{31-32}\), indicating that FVIIIA\(_1\) had also partially dissociated. This was more obvious when the protein associated with the Mono S gradient peak following a 2-h exposure to pH 8.0 conditions was analyzed by SDS-PAGE. Fig. 4, lane 4, shows only a single major band associated with FVIIIA\(_{31-32}\), indicating that the FVIIIA\(_1\) subunit of FVIIIA\(_{31-32}\) also completely dissociates upon prolonged exposure at pH 8.0.

Subunit dissociation of FVIIIA\(_{31-32}\) also was evaluated by analytical velocity sedimentation. FVIIIA\(_{31-32}\) was exposed to pH 8.0 for 1 h followed by readjusting the pH to 6.0 and analytical ultracentrifugation. At 1 h, 87% of the activity had disappeared. In the control run, NaOH and HCl were added simultaneously to simulate the additions without the pH perturbation. Fig. 5 compares the sedimentation velocity profiles of the two samples. The control sample (Fig. 5A) exhibited a single boundary associated with a sedimentation coefficient (s\(_{20w}\)) of 7.3 S consistent with previous results with FVIIIA\(_{31-32}\) (16). The pH-inactivated sample also showed a single boundary, but two differences from the control sample were noted. First, the s\(_{20w}\) was decreased to 5.6 S consistent with the presence of a small sedimenting species. Additionally, the plateau height of the absorbance, which is a direct measure of the amount of protein in the cell, was decreased by 20% in the sample that had been exposed to pH 8.0. This shows a significant loss of mass from the centrifuge cell, presumably due to precipitation of the dissociated subunit(s). The results of this experiment are consistent with the hypothesis that one or both heavy chain-derived subunits of FVIIIA\(_{31-32}\) are dissociated under the conditions of the experiment. The results also suggest that, once dissociated, the FVIIIA\(_2\) fragment is poorly soluble. This sedimentation analysis provided independent evidence for subunit dissociation in solution and argues against the possibility that the Mono S resin dissociates an intact, inactive heterotrimer by binding to and denaturing newly exposed binding sites on FVIIIA\(_1\) and FVIIIA\(_2\).

**DISCUSSION**

The experimental conditions used in this study were designated to isolate the effect of pH on the activity of FVIIIA\(_{31-32}\). The concentration of FVIIIA\(_{31-32}\) was greater than 0.05 mg/ml (3 \times 10^{-7} \text{ M}) to ensure stable activity at pH 6 (16). Equilibrium sedimentation analysis of FVIIIA\(_{31-32}\) indicates that the dominant species is the heterotrimer under these conditions (16). Under the conditions used in Fig. 1, greater than 99% of the activity is lost over a period of several hours when the pH is raised to 8.0. The activity cannot be recovered by decreasing the pH back to 6.0 (Fig. 2). Two independent experimental approaches indicate that the loss of activity is associated with dissociation of the heterotrimer. First, Mono S HPLC of FVIIIA\(_{31-32}\) exposed to pH 8.0 for different times results in the isolation of a species lacking the FVIIIA\(_2\) subunit followed by the isolation of a species lacking both the FVIIIA\(_1\) and FVIIIA\(_2\) subunits (Figs. 3 and 4). Second, velocity sedimentation of pH-inactivated FVIIIA\(_{31-32}\) reveals a marked decrease in the sedimentation coefficient and, interestingly, a decrease in the absorbance of sedimenting protein (Fig. 5). Since the sedimentation analysis was done under conditions where the Mono S experiment predicts that FVIIIA\(_1\) should be largely associated, the loss of sedimenting mass indicates that the FVIIIA\(_1\) subunit has precipitated from solution. This is consistent with the finding that FVIIIA\(_1\) can only be identified in trace amounts in unbound column fractions following Mono S chromatography (Fig. 4). Under the conditions used in Fig. 1, significant but incom-
Complete loss of activity of fVIIIa is detectable at pH 7.0 and 7.5. After decaying to the values shown at 6 h, the activity is stable for up to 72 h. The results are consistent with our previous results at pH 7.4 during either the activation of fVIII by thrombin or during stability testing studies of isolated fVIIIa at this pH (16). When fVIII (0.04 mg/ml) was activated with thrombin at pH 7.4, the initial peak activity declined approximately 50% after 1.5 h. Activation of lower concentrations of porcine fVIIIa is associated with a significantly greater loss of activity at this pH (10, 11). The initial loss of activity is not due to further proteolysis by thrombin (10, 11), but, over a period of 24 h, substantial degradation of the fVIIIa subunit occurs (16). Our initial results with isolated fVIIIa indicated that at pH 7.4 the preparation retains substantial stable activity. However, it is now clear that a major transition occurs at slightly greater pH values (Fig. 1, inset).

A model that is consistent with these data is shown in Fig. 6. The dominant pathway for the complete dissociation of the heterotrimer into individual subunits involves an intermediate, $\text{fVIII}_{\text{A2,C1,C2}}$, which was isolated in this study. This intermediate has no detectable activity. The equilibrium constant for the formation of this intermediate is pH-dependent. Following the dissociation of the fVIIIa subunit, an irreversible step, precipitation of the subunit, occurs. Interpreted literally, the irreversible step in the model predicts that a mixture of active and inactive forms cannot co-exist, since the precipitation of the fVIIIa fragment would drive the equilibrium to a completely inactive state. Yet at pH 7.0 and 7.5, an apparently stable, partially active preparation can be detected. One possible explanation is that precipitation of fVIIIa requires a critical concentration of fVIIIa monomers to occur. Failure to fully restore native structure by simply reversing the conditions (Fig. 2) has been observed frequently in oligomeric proteins and has been recently reviewed (21). During the process of reconstituting oligomeric proteins, “wrong aggregates” have been identified which represent the availability of folding pathways to non-native states (21). Although irreversible changes in the fVIIIa subunit appear to occur, the nature of the process is not understood and will require further investigation.

The denaturation of fVIIIa from the active heterotrimer to inactive dissociated species occurs over a narrow pH range, as shown in the inset in Fig. 1. This abrupt transition cannot be caused by the deprotonation of a single group, which would require an increase of 2 pH units to go from 90% to 10% protonation. Sharp, pH-induced transitions are indicative of cooperative processes and have been widely observed in studies of denaturation of proteins (22). The simplest model is that the protein exists in either of two equilibrium states, native and denatured. pH-dependent transitions result from a difference between the native and denatured protein in the $k_0$ values of ionizable groups (23). Application of this general concept to fVIIIa indicates that denaturation of one or more subunits occurs which then disrupts the quaternary structure

**Fig. 5.** Velocity sedimentation of pH-inactivated fVIIIa. fVIIIa, 0.1 mg/ml (6 x 10^-7 M) in 0.6 M NaCl, 5 mM Mes, 5 mM CaCl$_2$, 0.01% Tween 80, pH 6.0, underwent analytical velocity sedimentation at 260,000 x g as described under “Experimental Procedures.” A, stable fVIIIa at pH 6.0; B, the pH of the solution containing fVIIIa was increased to 8.0 by adding dilute NaOH, then readjusted after 1 h to 6.0 by adding dilute HCl. Six scans, obtained at 4-min intervals, are superimposed in the graph. The insets show the plots of the logarithm of the boundary position versus time and linear regression lines that were used to obtain the sedimentation coefficients.
coagulant activity was recovered from either column. The activity that emerged from Superose 13 correlated well with fractions that contained both $\text{fVIII}_{\text{A}}$ and $\text{fVIII}_{\text{A}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$. The $\text{fVIII}_{\text{A}}$ fragment eluted later than the heterodimer and contained no activity. Two different forms of the $\text{fVIII}_{\text{A}}$-$\text{A}_{\text{3}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ heterodimer appeared to emerge from the Mono Q column: a major form with no activity and a small amount of material with low but detectable activity. From these data, Fay concluded that the $\text{fVIII}_{\text{A}}$-$\text{A}_{\text{3}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ consists of a heterodimer. Since loss of activity was associated with no detectable dissociation of the heterodimer and occurred in the absence of thrombin, it was proposed that the loss of activity was due to a conformational change with the heterodimer.

Our subsequent study showed that the intact heterotrimer is much more active than the $\text{fVIII}_{\text{A}}$-$\text{A}_{\text{3}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ heterodimer (16). Although we are unable to detect any activity associated with this heterodimer, we cannot exclude the possibility that this species is active transiently. Thus, our conclusions do not contradict Fay's findings but emphasize the importance of the $\text{fVIII}_{\text{A}}$ subunit in maintaining the functional integrity of $\text{fVIII}_{\text{A}}$. In addition to dissociation of the $\text{fVIII}_{\text{A}}$ subunit, it appears that the $\text{fVIII}_{\text{A}}$ subunit dissociates after prolonged incubation under the conditions of our study. This dissociation step, which does not appear to be functionally important, was not observed by Fay.

Factor VIII is homologous to another plasma blood coagulation cofactor, factor V. Factor V also possesses the $\text{A}_{\text{1}}$-$\text{A}_{\text{2}}$-$\text{B}_{\text{1}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ domal sequence (24, 25). Like factor VIII, the activation of factor V by thrombin involves the dissociation of the entire B domain. However, there is no cleavage of factor V between the A1 and A2 domains. Thus, thrombin-activated factor V (factor Va) is a heterodimer. The isolation of various thrombin-derived proteolytic fragments of factor V followed by the successful reconstitution of activity at pH 7.5 in the presence of $\text{Ca}^{2+}$ or $\text{Mn}^{2+}$ originally established factor Va as a heterodimer (26). Subsequently, thermodynamic and hydrodynamic properties of the subunit interaction were studied extensively (27, 28). At pH 7.4-7.65, the subunits are associated tightly with a dissociation constant in the nanomolar range. Since the pH dependence of the subunit interaction has not been studied for factor Va, and since dissociation constants for the assembly of $\text{fVIII}_{\text{A}}$-$\text{A}_{\text{3}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ have not been determined, it is not possible to compare the relative stability of the cofactors. However, it is reasonable to propose that $\text{fVIII}_{\text{A}}$-$\text{A}_{\text{3}}$-$\text{C}_{\text{1}}$-$\text{C}_{\text{2}}$ is less stable than factor Va and that this results from weak association of the $\text{fVIII}_{\text{A}}$ subunit.

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