Is There a Precursory, Relatively Procoagulant-Inactive Form of Normal Antihemophilic Factor (Factor VIII)?

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When factor VIII/von Willebrand factor (FVIII/vWF) is chromatographed on 4% agarose in 0.25 M CaCl₂, the procoagulant activity partially separates from the void volume protein peak. If FVIII/vWF is reacted with thrombin prior to chromatography, both the magnitude and resolution of the procoagulant activity peak are greatly increased. These observations suggest that activated species of FVIII/vWF are contained in the late-eluting procoagulant activity peak, and therefore, a precursory form of FVIII/vWF may exist. Such a precursor might be expected to: (1) lack procoagulant activity; (2) elute in the void volume from 4% agarose-CaCl₂; and (3) be activated by thrombin, after which the procoagulant activity should elute in the inner volume from 4% agarose-CaCl₂ columns. The specific procoagulant activities for FVIII/vWF purified in the presence and absence of the protease inhibitors, heparin-bovine pancreatic trypsin inhibitor and diisopropylfluorophosphate, were 27 and 92 U/mg, respectively. For FVIII/vWF isolated in the presence of this mixture of inhibitors, we observed that a peak with very low specific procoagulant activity, but containing at least 80% of the total activity, consistently eluted in the void volume from 4% agarose-0.25 M CaCl₂ gel filtration. The FVIII/vWF prepared in the absence of protease inhibitors could be activated 13.8 ± 3.0-fold by thrombin, compared to 6.6 ± 1.7-fold for FVIII/vWF prepared without protease inhibitors. When FVIII/vWF prepared in the presence of inhibitors is activated by thrombin prior to 4% agarose-0.25 M CaCl₂ chromatography, virtually no FVIII procoagulant activity elutes in the void volume; instead, a considerably enhanced symmetrical peak of FVIII procoagulant activity elutes in the inner volume. These data imply that FVIII/vWF circulates in vivo as a precursor with little or no procoagulant activity until activated by thrombin or a thrombin-like enzyme.

Factor VIII/von Willebrand Factor (FVIII/vWF) is a large glycoprotein that elutes in the void volume from 2% to 6% agarose in dilute neutral buffers. Studies from many laboratories have demonstrated that 4% agarose gel filtration of FVIII/vWF in high ionic strength solvents, particularly 0.25 M CaCl₂, separates most of the FVIII procoagulant activity from the protein and vWF activity. We have recently shown that activation of human FVIII/vWF with thrombin prior to 4% agarose gel filtration in 0.25 M CaCl₂ enhances the content of FVIII procoagulant activity in the late-eluting peak and improves its resolution from the void volume protein. These studies prompted the hypothesis that FVIII/vWF might circulate in blood as a precursor with little or no FVIII procoagulant activity until exposed to thrombin or a thrombin-like enzyme.
Theoretically, such a precursor species should: (1) have no FVIII procoagulant activity; (2) elute in the void volume from 4% agarose in 0.25 M CaCl₂; and (3) be activated by thrombin to give a species with enhanced procoagulant activity that then elutes in the inner volume of a 4% agarose column in 0.25 M CaCl₂. To test this hypothesis directly, FVIII/vWF protein must first be prepared under conditions that minimize its becoming even partially activated and then examined for the fulfillment of the above three criteria. Others have reported that the inclusion of various mixtures of inhibitors during the purification procedure has not prevented the appearance of the late-eluting peak of FVIII procoagulant activity when the preparations are chromatographed on 4% agarose columns in 0.25 M CaCl₂.12,13 One group has shown that the use of bovine-pancreatic trypsin inhibitor in their isolation procedure prevented the late elution of FVIII procoagulant-active species from 4% agarose in 1 M NaCl.14 The interpretation of those experiments is uncertain, since, in contrast to chromatography in 0.25 M CaCl₂, some investigators have not been able to show that FVIII procoagulant activity separates from the void volume protein in 1 M NaCl even when protease inhibitors are not used.11,15

We have prepared human FVIII/vWF protein from fresh-frozen plasma in the presence and absence of several different inhibitor mixtures and now wish to report the chromatographic analyses of these preparations on 4% agarose columns in 0.25 M CaCl₂. Our results have relevance to the design of future experiments intended to define the structure–function relationships of the FVIII/vWF molecule.

**Materials and Methods**

**Materials**

All chemicals were reagent grade or better and were used without further purification. Heparin was purchased from the Upjohn Company (Kalamazoo, Mich.), Trasylol® (kallikrein inhibitor, bovine-pancreatic trypsin inhibitor) from FBA Pharmaceuticals (New York, N.Y.), soybean trypsin inhibitor from Worthington Biochemical Corp. (Freehold N.J.), and diisopropylfluorophosphate (DFP) from Sigma Chemical Co. (St. Louis, Mo.) or Calbiochem (San Diego, Calif.). Alumina C gel was obtained from Calbiochem, bentonite from Sigma, polyethylene glycol (Carbowax 4000 from Union Carbide Corp., New York, N.Y.), and 4% agarose (Biogel A-15m, 200–400 mesh) from Bio-Rad Laboratories (Richmond, Calif.).

**Determination of Protein**

Protein concentrations were estimated by the absorbance at 280 nm, corrected for light scattering at 320 nm. Experiments in our laboratory relating the absorbance at 280 nm to the protein concentration determined by the method of Lowry et al.16 give an E₁% of 12.3 for FVIII/vWF. Protein concentrations expressed as milligrams per milliliter have been converted from absorbance units using this extinction coefficient.

**Assays for FVIII Procoagulant Activity**

FVIII procoagulant activity was determined by the partial thromboplastin time method of Langdell et al.14 except that the hemophilic substrate plasma with <1% of normal activity was activated by incubation with kaolin for 6 min at 37°C. Our modifications of this assay for solutions containing calcium have been described in detail.10,11

**Isolation of FVIII/vWF From Intermediate Purity FVIII/vWF Concentrate**

The purification of FVIII/vWF, obtained from the American National Red Cross, was performed as we have previously reported,4,10,11 except that the first polyethylene glycol precipitation was done at a concentration of 6%. The solution was then adjusted to 12% polyethylene glycol concentration, and the
The FVIII/vWF precipitate that formed was redissolved in 0.05 M Tris-HCl-0.15 M NaCl and stored in aliquots at –20°C.

Purification of FVIII/vWF From Plasma in the Presence of Proteolytic Inhibitors

Since the first step in the purification of FVIII/vWF from plasma involves the formation of cryoprecipitate, we first compared the benefits of adding the inhibitors at the time of cryoprecipitation versus adding them at the time of blood collection. One combination of inhibitors added at the time of blood collection was sufficiently effective such that the addition of inhibitors at other steps in the purification procedure was not examined. The inhibitors used were heparin (2500 U/liter of blood) with soybean trypsin inhibitor (SBTI; 15 mg/liter of blood) or bovine-pancreatic trypsin inhibitor (BPTI; 100,000 kallikrein inactivator U/liter of blood). No other additions of these inhibitors were made during the purification procedure, since further additions appeared to interfere with the fractionation steps. Diisopropylfluorophosphate (DFP) was also used as an inhibitor throughout the purification procedure. Initially, to minimize the amount added, DFP was added only twice: when the cryoprecipitate was redissolved and when the second polyethylene glycol precipitate was redissolved prior to gel filtration. As will be shown later, the recovery of FVIII procoagulant activity in the void volume was much better when DFP was also added as the frozen plasma was thawed at 4°C to form the cryoprecipitate. It was not routinely added to the whole blood, because it seemed to promote red cell hemolysis.

In our laboratory, the best conditions for the purification of “precursor” FVIII/vWF were to collect 430 ml blood from each donor in commercially available plastic blood collection bags that contained 70 ml acid citrate dextrose anticoagulant and heparin; BPTI or SBTI was then added to the bag as soon as the needle was removed from the donor’s arm. The blood was centrifuged immediately for 15 min at 3500 g and 4°C and the plasma collected in transfer packs and promptly frozen at –90°C. The entire blood collection process from collection to placement in the freezer was completed within 30 min. The formation of cryoprecipitate from ~600 ml of plasma from 2–3 donors was begun on the same day that the blood had been collected. The purification procedure was then performed continuously until the 4% agarose-0.25 M CaCl₂ chromatographic step had been completed. The purification method was that of Newman et al., with the exception that a bentonite adsorption step was used as we have previously described. In addition, DFP was added at three points in the purification process: (1) when the plasma had been thawed to slush and transferred to a beaker, it was made 1 mM in DFP; (2) after the cryoprecipitate had been harvested by centrifugation and washed with the ice-cold 8% ethanol, it was redissolved in buffer that had been made 2.5 mM in DFP; and (3) after the final polyethylene glycol precipitation step, the precipitate was recovered by centrifugation, washed, and then redissolved in buffer made 2.5 mM in DFP. This latter solution was then immediately chromatographed at room temperature on a 4% agarose column (1.5 x 32 cm) in 0.05 M Tris-HCl-0.15 M NaCl, pH 7.35. The fractions eluting in the void volume were pooled and chilled to 4°C. Then an equal volume of ice-cold 40% polyethylene glycol in 0.05 M Tris-HCl-0.15 M NaCl was added to quantitatively precipitate the small amount of protein. After being stirred for 15 min at 4°C, the mixture was centrifuged for 10 min at 6000 g. The purified FVIII/vWF precipitate was dissolved in 0.5–1.0 ml Tris-HCl-0.15 M NaCl, pH 7.35. Then 9 volumes of this FVIII/vWF solution were mixed with 1 volume of 2.5 M CaCl₂ in 0.05 M Tris-HCl-0.15 M NaCl and the solution immediately chromatographed on 4% agarose in 0.25 M CaCl₂, as described next.

Chromatography of Purified FVIII/vWF and Thrombin-Activated FVIII/vWF on 4% Agarose in 0.25 M CaCl₂

Separate plastic chromatography columns (0.9 x 11 cm) of 4% agarose were used for the rechromatography of the purified FVIII/vWF before and after thrombin activation. In each experiment, a 0.5-ml sample was applied directly to the top of the agarose. The column had been equilibrated with 0.05 M Tris-HCl-0.25 M CaCl₂-0.01 M NaCl, pH 7.35, and was developed with the same solvent at a flow rate of ~5 ml/hr; fraction volumes of ~0.45 ml were collected. For the chromatography of thrombin-activated FVIII/vWF, a sample of the purified FVIII/vWF in 0.05 M Tris-HCl-0.15 M NaCl was
activated with purified human thrombin (Bureau of Biologics, FDA, specific activity 500 NIH U/mg) at a final concentration of 0.04 U/ml. After incubating for 10 min at room temperature (~25°C), the reaction mixture was adjusted to 0.25 M CaCl₂ by the addition of 2.5 M CaCl₂ and then chromatographed on a 4% agarose column (0.9 x 11 cm) exactly as for the control. Recovery of FVIII/vWF from the columns averaged ~80%, which is comparable to the yields that we have previously reported for FVIII/vWF prepared from lyophilized concentrates and chromatographed on 4% agarose in 0.25 M CaCl₂ under similar conditions.

**Determination of Specific Activity and Degree of Activation by Thrombin**

Special precautions were taken to ensure the accuracy of the comparisons of specific activities and extents of activation for the FVIII procoagulant function. FVIII/vWF samples to be used for specific activity determinations were assayed at dilutions that gave clotting times within the most sensitive range of our assay, 90–150 sec (buffer control ≥ 250 sec). The protein concentration of FVIII/vWF samples used for thrombin activation experiments was ~0.5 absorbance U. Aliquots of this solution were assayed before and after the addition of thrombin at the appropriate dilution; hence, the FVIII/vWF to thrombin ratio remained constant at each dilution. Under our assay conditions, thrombin did not affect the clotting time.

**RESULTS**

**FVIII/vWF Purified From Lyophilized Concentrates**

In our laboratory, the customary starting material for the purification of FVIII/vWF has been intermediate-purity FVIII/vWF concentrates. When highly purified FVIII/vWF prepared from these concentrates is chromatographed on 4% agarose in 0.25 M CaCl₂, we always observed results similar to those reported from many other laboratories, i.e., essentially all of the protein and vWF activity are eluted in the void volume, but most of the FVIII procoagulant activity is eluted later. With one preparation of FVIII/vWF purified from these concentrates, however, duplicate experiments showed that the protein elution profile was the same as usual, but the peak of FVIII procoagulant activity consistently eluted in the void volume with some trailing of activity into the inner volume (Fig. 1A). Activation of the sample with thrombin before chromatography on 4% agarose in 0.25 M CaCl₂ resulted in a complete shift of FVIII procoagulant activity, now enhanced about twofold, to a much later elution position (Fig. 1B). This latter chromatogram of thrombin-activated FVIII/vWF is in good accord with our deductions about the chromatography of FVIII/vWF, which in retrospect must have contained partially activated FVIII/vWF species.

**FVIII/vWF Purified From Plasma in the Presence of Inhibitors**

We thought that the unusual chromatographic behavior of the above preparation of FVIII/vWF, to which no inhibitors had been added at any step, might have been due to the isolation of a greater than usual amount of nonactivated FVIII. Conceivably, this could have been due to a fortuitous lack of FVIII/vWF activation—such as by trace amounts of thrombin or a thrombin-like enzyme—during blood collection and/or during the preparation of the intermediate-purity concentrate. To test this hypothesis, FVIII/vWF preparations were purified from plasma in the presence of different combinations of proteolytic inhibitors and then gel filtered on 4% agarose-0.25 M CaCl₂ columns. The percent of recovered FVIII procoagulant activity in the void volume was the initial criterion used to evaluate the effectiveness of each inhibitor combination, since this parameter seemed less subject to random variations than either specific activity or extent of activation by
thrombin. Table 1 lists the percent of recovered FVIII procoagulant activity eluting in the void volume for each FVIII/vWF preparation. Several points emerge from the data in Table 1. First, except for the addition of SBTI and heparin during cryoprecipitate preparation, all of the inhibitor combinations tested were associated with an increase in the amount of FVIII procoagulant activity eluting in the void volume. Second, FVIII/vWF prepared from blood to which inhibitors had been added during and immediately after collection, and hence before freezing the plasma, had much more FVIII procoagulant activity in the void volume than FVIII/vWF prepared from plasma to which inhibitors were first added during the

Table 1. Effect of Protease Inhibitor Combinations on the Amount of Recovered FVIII Procoagulant Activity Eluting in the Void Volume From 4% Agarose Columns in 0.25 M CaCl₂

<table>
<thead>
<tr>
<th>Inhibitor Combination</th>
<th>Time of SBTI-Heparin or BPTI-Heparin Additions</th>
<th>Percent of Total FVIII Procoagulant Activity in Void Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitors present</td>
<td>CP</td>
<td>25§</td>
</tr>
<tr>
<td>SBTI*-heparin; 2 DFP* additions†</td>
<td>CP†</td>
<td>30</td>
</tr>
<tr>
<td>BPTI*-heparin; 3 DFP additions</td>
<td>CP</td>
<td>45§</td>
</tr>
<tr>
<td>SBTI-heparin</td>
<td>WB†</td>
<td>45</td>
</tr>
<tr>
<td>SBTI-heparin; 2 DFP additions</td>
<td>WB</td>
<td>54</td>
</tr>
<tr>
<td>SBTI-heparin; 3 DFP additions</td>
<td>WB</td>
<td>65</td>
</tr>
<tr>
<td>BPTI-heparin; 3 DFP additions</td>
<td>WB</td>
<td>80§</td>
</tr>
</tbody>
</table>

*SBTI, soybean trypsin inhibitor; BPTI, bovine-pancreatic inhibitor; DFP, diisopropylfluorophosphate.
†CP, cryoprecipitation step; WB, whole blood.
††See Materials and Methods section for the steps at which the DFP additions were made during the purification.
§Mean of 3 separate purifications, using 6-9 different donor plasmas.
cryoprecipitation step. Third, BPTI was a more effective inhibitor than SBTI, consistent with its having a higher affinity for proteases. Fourth, the most effective inhibitor combination tested was the addition of BPTI to the citrated-heparinized blood just after blood collection and then the addition of DFP at the three steps described in the Materials and Methods section. With this combination of inhibitors, 80% of the FVIII procoagulant activity, albeit very low specific activity, eluted in the void volume from 4% agarose in 0.25 M CaCl₂. Also apparent in Table 1 is the importance of adding DFP as early as possible in the isolation of FVIII/vWF. Plasma was separated from heparinized blood to which SBTI had been added just after collection; then, DFP was either not used at all or added at 2 or 3 selected steps during the plasma fractionation. When no DFP was used, the void volume contained 45% of the total FVIII/vWF activity recovered during chromatography on 4% agarose in 0.25 M CaCl₂; however, with 2 and 3 DFP additions, 54% and 65%, respectively, of the total eluting FVIII/vWF activity was recovered in the void volume. Of interest, one fractionation of FVIII/vWF in which DFP was added 3 times, and was the only inhibitor used, gave about the same amount of FVIII procoagulant activity (51%) in the void volume as FVIII/vWF prepared from plasma to which heparin, BPTI, and DFP were added beginning with cryoprecipitation. Central to our interpretation of the data is our ability to vary the amount of FVIII procoagulant activity in the void volume by varying the inhibitors chosen. Thus, without inhibitors or with certain inhibitor combinations, most of the FVIII procoagulant activity eluted in the inner column volume, as it did in several other reports in which inhibitors were used. For example, the void volume activity that we obtained using SBTI and heparin, but no DFP, is comparable to that obtained by Poon and Ratnoff using an inhibitor mixture that also contained SBTI and heparin, but no DFP. Also, the results of our experiments in which the inhibitors were added at the time of cryoprecipitation, like those of Sussman and Weiss, also did not prevent the late elution of the FVIII procoagulant activity. Thus, it is all the more striking that the addition of BPTI, heparin, and DFP at the time of blood collection allowed us to isolate an FVIII/vWF species with 80% activity in the void volume from 4% agarose-0.25 M CaCl₂ columns.

It should be emphasized that the increase in the percent activity recovered in the void volume was not due to variations in the protein loads. All of the experiments were done using a protein concentration of ~0.8 absorbance unit or less, such that the peak of FVIII procoagulant activity should have eluted at a volume of at least twice the void volume. Thus, instead of promoting association of the FVIII procoagulant activity with the void volume protein, our experimental conditions actually favored dissociation. Despite this, the FVIII procoagulant activity did not shift from the void volume to a later position in the chromatogram, even when very low concentrations of the FVIII/vWF prepared with the optimal combination of inhibitors were filtered on the 4% agarose-0.25 M CaCl₂ columns.

Effect of Thrombin on Purified "Precursor" FVIII

Figure 2A presents the 4% agarose-0.25 M CaCl₂ gel filtration pattern of an aliquot of FVIII/vWF prepared from blood containing heparin and SBTI, the plasma from which was then fractionated in the presence of three DFP additions. For this experiment, FVIII/vWF with a protein concentration of 0.774 was made
Fig. 2. Chromatography of control (A) and thrombin-activated "precursor" (B) FVIII/vWF on a 4% agarose column (0.9 x 11 cm) in 0.25 M CaCl₂. The protein profiles of both chromatograms are similar and consist of a single sharp peak in the void volume. Prior to thrombin activation, the FVIII procoagulant activity eluted essentially in the void volume. Thrombin activation enhanced the FVIII procoagulant activity 18.6-fold and caused it to elute as a single sharp peak much beyond the void volume.

0.25 M in CaCl₂ prior to chromatography. The protein and FVIII procoagulant activity eluted almost simultaneously in the void volume, with the FVIII procoagulant peak slightly trailing the protein peak. When another aliquot of FVIII/vWF prepared in the presence of the same inhibitors was incubated in Tris-HCl-NaCl with 0.04 U thrombin/ml, the FVIII procoagulant activity was enhanced 18.6-fold. The thrombin-activated FVIII/vWF solution was then adjusted to 0.25 M CaCl₂ and chromatographed on 4% agarose in 0.25 M CaCl₂ to give the elution pattern shown in Fig. 2B. Just as reported earlier for thrombin-activated FVIII/vWF prepared from lyophilized concentrates, the greatly enhanced FVIII procoagulant activity eluted as a fairly sharp peak considerably displaced from the void volume.

If the FVIII/vWF molecule does in fact exist in a precursor form, such a molecular species should have little or no FVIII procoagulant activity unless activated. Hence, FVIII/vWF isolated under optimal conditions with respect to proteolytic enzyme inhibition ought to have a lower specific procoagulant activity than FVIII/vWF purified under the sorts of conditions usually reported. Moreover, thrombin should activate such a precursor species to a much greater extent than the FVIII/vWF prepared using little or no proteolytic inhibition. As shown in Table 2, the FVIII/vWF purified from plasma in the absence of inhibitors had a specific activity of 92 U/mg and could be activated 6.6-fold by thrombin. When purified in the presence of the best combination of inhibitors tested, the specific activity was only 27 U/mg but could be enhanced 13.8-fold by thrombin. The standard curves of procoagulant activity of FVIII/vWF preparations, whether purified in the absence or presence of inhibitors, were parallel to that for FVIII/vWF purified from lyophilized concentrates. While the half-life for the inactivation of thrombin-activated FVIII/vWF from plasma without inhibitors was about the same as that from concentrates, thrombin-activated FVIII/vWF purified in the presence of inhibitors was a little more stable. At the present time, this latter
Table 2. Thrombin Activation of FVIII/vWF Prepared in the Absence and Presence of Inhibitors During Purification: the Values are the Means ± SEM for Three Separate Experiments Using Different Plasma Samples Each Time

<table>
<thead>
<tr>
<th>Inhibitors Used</th>
<th>Specific Activity (U/mg)</th>
<th>Enhancement of FVIII Procoagulant Activity by Thrombin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>92 ± 23</td>
<td>6.6-fold ± 1.7</td>
</tr>
<tr>
<td>BPTI* -heparin before freezing;</td>
<td>27 ± 14</td>
<td>13.8-fold ± 3.0</td>
</tr>
<tr>
<td>3 DFP* additions</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*BPTI, bovine-pancreatic trypsin inhibitor; DFP, diisopropylfluorophosphate.  
†Final thrombin concentration: 0.04 U/ml.

difference is difficult to interpret and will require further study, since the kinetics of inactivation appear to be very complicated. Despite this limitation, however, our studies are consistent with the interpretation that a precursor form of FVIII/vWF can be isolated when proteolytic enzyme inhibition is maximized in freshly collected blood and then maintained during the purification procedure.

**Examination of Thrombin-Activated FVIII/vWF for Serine Protease Activity**

Two types of experiments were designed to test the possibility that thrombin-activated human FVIII/vWF might give rise to a serine protease similar to that reported by Vehar and Davie for thrombin-activated bovine FVIII. In one experiment, DFP was added to a final concentration of 2.5 mM at the time of maximum activation of FVIII by thrombin. Figure 3 shows that thrombin-activated FVIII lost activity with time, but that the rate of inactivation was the same whether or not DFP was present. Similarly, 2.5 mM DFP had no effect on the FVIII procoagulant activity of thrombin-activated FVIII/vWF in 0.25 M CaCl₂, either before or after separation by 4% agarose chromatography in that solvent. Thus, in contrast to the results of Vehar and Davie, DFP had no effect on the FVIII procoagulant activity of the thrombin-activated human FVIII/vWF protein. If activation of FVIII/vWF by thrombin indeed gave rise to a serine protease, one might expect an enhancement in the overall proteolytic activity of the solution. Using the chromogenic substrate N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide, which is known to be broadly susceptible to a variety of serine proteases, no enhancement of amidase activity could be detected during a 1.5-hr incubation of FVIII/vWF with thrombin and the synthetic substrate. These
experiments suggest that, at least within the limitations of the two methods used, activation of human FVIII/vWF by thrombin does not generate serine protease or peptidase activity; moreover, incubation of FVIII/vWF with thrombin does not appear to enhance the proteolytic capabilities of thrombin.

**Partially Activated FVIII/vWF**

In the strictest sense, precursor FVIII/vWF protein should possess no FVIII procoagulant activity until activated. Yet FVIII/vWF, as ordinarily isolated from plasma, has FVIII procoagulant activity; therefore, we reasoned that activation by thrombin or a thrombin-like protease might have occurred during the collection or processing of the blood. The fact that such purified FVIII/vWF preparations can be activated further by thrombin suggests that either nonactivated or partially activated forms of FVIII/vWF are present. To test this hypothesis, we attempted to progressively activate FVIII/vWF prepared from lyophilized concentrates. FVIII/vWF, at a protein concentration of 0.76 absorbance U, was activated at 0.5° with 0.02 U thrombin/ml. Under these conditions, the activated state of FVIII/vWF remained at a constant level for about 1 hr. At intervals of about 6 min, the thrombin concentration was increased by 0.02 U/ml to give final thrombin concentrations of 0.04, 0.06, and 0.08 U/ml. Figure 4 shows that the procoagulant activity of FVIII/vWF increased in a step-wise fashion and that the activity levels attained for a final thrombin concentration were about the same whether the thrombin had been added sequentially with time or all at once. These results are consistent with the existence of partially activated forms of FVIII/vWF or of a mixture of activated and nonactivated species in FVIII/vWF as usually purified. Our previous observations that the FVIII procoagulant activity isolated from the 4% agarose-0.25 M CaCl₂ chromatography of thrombin-activated FVIII/vWF can itself be further activated by thrombin supports the former interpretation. Either model is consistent with the established observation that procoagulant-active FVIII/vWF, as usually purified in the absence of protease inhibitors, can be

![Figure 4. Stepwise activation of FVIII/vWF by thrombin at 0.5°C. FVIII/vWF at a concentration of 0.76 absorbance U was activated by 4 successive additions of thrombin at 0, 6, 12, and 18 min to give final concentrations of 0.02, 0.04, 0.06, and 0.08 U thrombin/ml. Experiments in which 0.04 or 0.08 U thrombin/ml of solution was added all at once are shown in the shaded bars. Whether thrombin was added stepwise or as a single bolus, about the same levels of FVIII procoagulant activity were observed at the same final thrombin concentrations.](image)
factor VIII conversion to factor VIII

Further activated by thrombin. The relatively low levels of activation in Fig. 4 are probably due to the reduced temperature used in the experiment and to the use of FVIII/vWF purified from lyophilized concentrates.

**DISCUSSION**

Our results demonstrate that it is possible to prepare human FVIII/vWF such that the FVIII procoagulant activity elutes in the void volume from 4% agarose in 0.25 M CaCl₂. This occurred best with preparations of FVIII/vWF prepared from heparinized blood to which BPTI was added as soon as the needle was removed from the donor's arm. After the plasma was separated and frozen, DFP was added during the formation of the cryoprecipitate and then twice again during the remaining fractionation steps. Under these conditions, 80% of the FVIII procoagulant activity consistently eluted in the void volume from 4% agarose columns in 0.25 M CaCl₂. Our results show not only that heparin-BPTI-DEP is the best combination of inhibitors tested, but also that it is important to add these inhibitors as early as possible.

About 25 yr ago, Quick and his colleagues suggested that the expression of antihemophilic activity depended on the activation of a precursor by thrombin; subsequently, Rapaport and coworkers confirmed and extended these observations and made the same suggestion. Our results now lend further support and expand this notion. That we are describing a precursive form of FVIII/vWF seems likely for three reasons: (1) the specific activity of the procoagulant function of FVIII/vWF prepared from plasma in the presence of protease inhibitors is considerably lower than that prepared in the absence of inhibitors; (2) at least 80% of its FVIII procoagulant activity elutes in the void volume from 4% agarose in 0.25 M CaCl₂; and (3) FVIII/vWF prepared using the best combination of protease inhibitors tested can be activated to a greater extent by thrombin than FVIII/vWF purified without protease inhibitors, and after thrombin activation, the enhanced peak of FVIII procoagulant activity then elutes as a sharp peak in the inner volume from 4% agarose in 0.25 M CaCl₂. Unlike thrombin-activated bovine FVIII/vWF, thrombin-activated human FVIII/vWF does not appear to be DFP-sensitive.

Our results have implications to the present knowledge about the structure-function relationships of the human FVIII/vWF molecule(s). First, the observations reported here are in line with our previous suggestion that the FVIII procoagulant activity that elutes late from 4% agarose-0.25 M CaCl₂ columns is an activated species of FVIII/vWF. Central to this interpretation is that a truly precursive species of FVIII/vWF should be demonstrable. We show that a FVIII/vWF species with "precursor" features behaves as a large species on 4% agarose-0.25 M CaCl₂ chromatography. Under those same conditions, its reduced content of FVIII procoagulant activity elutes in the void volume and without evidence of any late-eluting peak of FVIII procoagulant activity unless activated by thrombin prior to chromatography. We acknowledge that our present work does not settle the controversy about the structure of the FVIII/vWF protein(s) after thrombin activation. Three possibilities exist: (1) thrombin may cleave FVIII/vWF protein to release a peptide fragment that contains the FVIII procoagulant activity function; (2) thrombin may cleave one or both components of an FVIII/vWF complex, such that a conformational change causes the two components to dissociate; or (3) thrombin may cleave the precursor form of FVIII/vWF in one or
more places to give a derivative with FVIII procoagulant activity. This latter interpretation is consonant with our previous suggestion that activation gives rise to a proteolytically modified FVIII/vWF molecule that contains both FVIII and vWF activities and is composed of an undetermined number of covalently bonded, albeit proteolytically cleaved, subunits.10,11

The second implication of our studies is that human FVIII/vWF may be much more susceptible to activation by thrombin than bovine FVIII/vWF, since the human form appears to require a greater degree of protection from proteases during its isolation. One group has isolated an apparently precursive form of bovine FVIII/vWF from slaughterhouse blood collected into sodium oxalate, heparin, and soybean trypsin inhibitor.2 The method of blood collection in a slaughterhouse should be more likely to generate proteolytic activity prior to the contact of the blood with the inhibitor mixture than in the case of blood collected by venipuncture. Moreover, our studies suggest that their mixture of inhibitors should be less potent than that used in our study. However, in spite of conditions that would seem likely to result in diminished FVIII procoagulant potential, their purified bovine FVIII/vWF could be activated 120-fold by thrombin that is much more than reported for the human molecule.15 Hence, we suggest that bovine FVIII/vWF is more resistant than human FVIII/vWF to proteolytic cleavage during purification. Also compatible with the above reasoning is that their bovine FVIII/vWF preparations could not be dissociated on 4% agarose in 0.25 M CaCl₂ until activated by thrombin.21

Our results clearly indicate that without extreme precautions, human FVIII/vWF has already been partially activated when isolated. It is interesting to note the obvious functional analogies between FVIII/vWF and factor V: neither appears to be an enzyme, yet both greatly enhance the reaction rates of certain blood-clotting serine proteases. Only recently has factor V been isolated as a single-chain precursive species that is cleaved by thrombin with an accompanying marked increase in activity.24 Like factor V, human FVIII/vWF may exist in vivo in a more precursive state than previously thought, and its conversion to an active form probably depends on thrombin or a thrombin-like enzyme.

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REFERENCES

7. Rick ME, Hoyer LW: Immunologic studies
FACTOR VIII CONVERSION TO FACTOR VIII


Is there a precursive, relatively procoagulant-inactive form of normal antihemophilic factor (factor VIII)?

ME Switzer, SV Pizzo and PA McKee