von Willebrand Factor Is a Cofactor for Thrombin-catalyzed Cleavage of the Factor VIII Light Chain*

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The proteolytic activation of highly purified, heterodimeric porcine factor VIII and factor VIII-von Willebrand factor complex by thrombin was compared at I 0.17, pH 7.0, 22 °C. During the activation of factor VIII, heavy-chain cleavage is necessary to activate the procoagulant function, whereas light-chain cleavage is required to dissociate factor VIII from von Willebrand factor complex. The kinetics of activation of free factor VIII and factor VIII-von Willebrand factor complex were identical. The steady-state kinetics of thrombin-catalyzed heavy-chain cleavages and light-chain cleavage of factor VIII either free or in complex with von Willebrand factor were studied using sodium dodecyl sulfate-polyacrylamide gel radioelectrophoresis and scanning densitometry of fragments derived from 125I-labeled factor VIII. Association of factor VIII with von Willebrand factor resulted in an 8-fold increase in the catalytic efficiency (kcat/Km) of light-chain cleavage (from 7 × 1010 to 54 × 1010 M−1 s−1). The catalytic efficiencies of heavy-chain cleavage at position 372 (≈ 6 × 109 M−1 s−1) and position 740 (≈ 100 × 109 M−1 s−1) were not affected by von Willebrand factor. We conclude that von Willebrand factor promotes cleavage of the factor VIII light chain by thrombin which is followed by rapid dissociation of the complex, so that the rate-limiting step becomes heavy-chain cleavage at position 372. This accounts for the observation that von Willebrand factor has no effect on the kinetics of activation of factor VIII by thrombin.

EXPERIMENTAL PROCEDURES

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

Materials—Hepes and Mes were purchased from BDH Chemicals. The W-3-3 hybridoma cell line producing mouse monoclonal anti-porcine factor VIII used for immunoaffinity chromatography was generously provided by Dr. D. N. Fass, Mayo Clinic/Foundation. W-3-3 antibody was coupled to Sepharose 4B at a concentration of 6 mg/ml as described previously (6). Carrier-free Na125I was purchased from Amersham. Lactoperoxidase and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PFP-Arg) were purchased from Behring Diagnostics. Lactoperoxidase was diluted to 20 IU/ml and stored at −70 °C in 0.2 M sodium acetate, 5 mM calcium nitrate, pH 6.8. Recombinant deoxylatehiedrin was a generous gift from Dr. R. B. Wallis, Ciba-Geigy Pharmaceuticals. Soybean trypsin inhibitor was purchased from Sigma. Spectrozyme FXa was purchased from American Diagnostics. Small, unilamellar phosphatidylcholine/phosphatidylserine vesicles (75/25%) w/w) were prepared by modification (7) of the method of Barenholz et al. (8). Other materials were obtained as described (9).

Isolation of Proteins—Plasma-derived porcine factor VIII was isolated as described previously (10). Porcine factor X was isolated as described previously (11) except that dextran sulfate-Sepharose chromatography was omitted. Porcine thrombin (11), vWF (10), vWf (12), and human vWF (13) were isolated as described previously. Factor IXa was isolated as described previously (11) with the following modifications. Heparin-Sepharose chromatography was carried out at 4 °C using 0.01 M Mes, pH 6.0, instead of 0.02 M Tris, pH 7.4. Additionally, soybean trypsin inhibitor (0.01 mg/ml) was added prior to heparin-Sepharose chromatography. The column was washed with 1 column volume of buffer containing soybean trypsin inhibitor prior to gradient elution of factor IXa. These modifications were made to minimize proteolytic degradation of the factor IXa heavy chain.

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‡The abbreviations used are: fVIII, factor VIII; vWF, von Willebrand factor; Mes, 2-(N-morpholino)ethanesulfonic acid; Hepes, (2-hydroxyethyl)-1-piperazineethanesulfonic acid; PFP-Arg, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; SDS-PAGE or SDS-PAGRE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis or radioelectrophoresis.

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Published extinction coefficients at 280 nm for porcine FVIII (14), FVIIIc (13), factor IXa (11), factor X (11), vWF (14), thrombin (11), and human vWF (13) were used.

Radioiodination of FVIII and FVIIIc-*.High-purified FVIII was labeled with [125I] by using a modification of the method of Thorell and Johansson (15) as follows. FVIII consisting of a mixture of 186/92-, 130/55-, and 55/30-kDa heterodimers in 0.1 M NaCl, 0.02 M Hepes, 5 mM CaCl2, 0.01% vWF were included as described in the Figure legends and the Table. Tween 80, pH 7.0. FVIII, 11.1-labeled fVII1, thrombin, and porcine with the following modifications. Spectrozyme FXa (0.4 mM) was
diography of dried gels and were quantitated using a Shimadzu CS- peroxidase to 2 units/ml for 3 min. The reaction was stopped by the addition of sodium azide to 0.1% (w/v) to inhibit lactoperoxidase. vWF was included during the reaction to protect against labeling the FVIIIc in the region of the vWF-binding site, since preliminary experiments showed that FVIII preparations labeled in the absence of vWF showed a reduction in vWF binding. Factor VIII was dissociated from vWF with Mg++ by the addition of 1 part 0.5 M NaCl, 1.275 M MgCl2, 0.5 to 4 parts of reaction mixture to the magnesium concentration exceeding the concentration of vWF. The mixture was added to W3/ Sepharose (1 ml) equilibrated in 5 mM Mes, 0.5 mM CaCl2, 0.01% Tween 80, pH 6.0 (W3 buffer), plus 0.1 M NaCl, 0.255 M MgCl2, at 20 ml/h, washed with 3 column volumes of W3-3 buffer plus 2 M NaCl, and then washed with W3-3 buffer plus 0.1 M NaCl until the radioactivity was base line (10-20 column volumes). [125I]-Labeled FVIIIc was eluted with W3-3 buffer plus 50% ethylene glycol (v/v), 1 M NaCl, 0.1% bovine serum albumin and stored at 4 °C before further use. The preparation was stable for at least 2 weeks.

[125I]-Labeled-FVIII was further purified by using Mono-Q high performance liquid chromatography as described for unlabeled FVIII except (1) at pH 7.4 (less as above), Fractions that contained predominantly 167/65-kDa heterodimer were used for further study. The concentration of [125I]-labeled-FVIII was determined by absorbance at 280 nm. [125I]-Labeled-FVIII contained 0.04-0.02 atoms (range of several preparations) of [125I]/FVIII molecule resulting in a specific radioactivity of 0.6-3 × 10^8 dpm/mg. The clotting activity of [125I]-Labeled FVIII from Mono-Q chromatography determined by single-stage assay using human hemophilia A plasma was not significantly different from unlabeled FVIII (80-190 units/mg) (6)). Additionally, activation of the product by thrombin, determined by two-stage clotting assay (1), was different from unlabeled FVIII. The coagulant activity of [125I]-labeled FVIII was stable for at least 2 weeks. This was verified by reasaying for clotting activity and by rechromatography on Mono-Q which yielded [125I]-labeled-FVIII eluting in the same position as the starting material.

FVIIIc was dialyzed into radioiodination buffer and then radiolabeled at a concentration of 0.1 mg/ml using the same conditions as described above except that vWF was omitted from the reaction. [125I]-Labeled FVIIIc was obtained by S-Sepharose chromatography. The column was equilibrated in 0.1 M NaCl and eluted with 0.8 M NaCl, 0.01 M histidine, 0.1% bovine serum albumin, pH 6.0.

Activation of FVIII and FVIIIc- vWF Complex by Thrombin—The kinetics of activation of FVIII were evaluated both by using a functional, plasma-free assay for activated FVIII and by analysis of proteolytic fragmentation of FVIIIc by SDS-PAGE as described below. Fractional cleavages were calculated by fitting to the Michaelis-Menten equation. The method of Lee and Wilson (19) was used to measure the velocity of cleavage corresponding to the absorption at each concentration. This method provides accurate estimates (<10% systematic error) of kcat and under conditions in which up to 50% of substrate is hydrolyzed. Velocity measurements were determined using a single time point. The fractional cleavage at positions 372 and 1689 was less than 0.3 in all cases. However, B domain cleavages approached 70% at high substrate concentrations so that the estimated kinetic parameters underestimate the true catalytic efficiency to some extent. Fits to the Michaelis-Menten equation were done by using the Marquardt algorithm with error estimates (expressed as S.D.) as described by Bevington (20).

Cleavage of FVIIIc, and FVIIIc-vWF Complex by Thrombin—The kinetics of cleavage of isolated [125I]-labeled FVIIIc (1 nM) in the presence and absence of porcine vWF and thrombin concentrations ranging from 5 to 150 nM in 0.15 M NaCl, 0.02 M Tris-Cl, 5 mM CaCl2, 0.01% Tween 80, pH 7.4, were studied. At each thrombin concentration, reactions were stopped at 15-5 intervals from 15 to 75 s by the addition of PPRCK to 1 μM. The fractional cleavage of [125I]-labeled FVIIIc was determined by using SDS-PAGE and scanning densitometry. The semilogarithmic dependence of cleavage on time yielded a first-order rate constant, kobs, at each thrombin concentration. Under conditions of excess enzyme, the dependence of kobs on thrombin concentration can be solved analytically to determine the steady-state parameters, kcat, and kobs (21), as described previously (18) (Equations 5 and 7 with η = 0).

RESULTS

Kinetics of FVIII Activation by Thrombin in the Presence and Absence of vWF—Thrombin was used to activate FVIII in either the presence or absence of a 2.5-fold molar excess of vWF, where the concentration of vWF refers to the concentration of subunits in the multimeric preparation (Fig. 1). Under these conditions FVIII is essentially completely complexed to vWF (14). It is apparent that free FVIII and FVIIIc-vWF complex activate at the same rate, reach the same peak activity, and decay at the same rate. The decay of FVIII, which is invariably observed at these concentrations, is due to a nonproteolytic event (10, 11) and may involve dissociation of the FVIIIa subunit (9). Increasing the concentration of vWF 5-fold had no effect on the kinetics of the reaction, indicating that the kinetics are independent of the fractional saturation of FVIII with vWF (data not shown).

Since vWF does not affect the kinetics of activation of FVIII, it is possible to draw some inferences based on earlier observations. Activated FVIII must dissociate from vWF before it can participate in factor X activation (5). The dissociation of FVIII from vWF is due to cleavage at position 1689 in FVIIIc to yield FVIIIa, C1-C2 which does not bind vWF (13). Since the
activation of fVIII-vWF complex by thrombin is as rapid as that of free fVIII, this "releasing cleavage" is not a rate-limiting step in the activation of fVIII-vWF complex. Additionally, it follows that subsequent dissociation of the cleaved product is not rate-limiting either.

Cleavage events accompanying the activation of free fVIII and fVIII-vWF complex were evaluated by SDS-PAGE (Fig. 2). It is evident by inspection of the autoradiograph that the rate of cleavage of fVIII, is increased considerably when fVIII is complexed to vWF. In contrast, cleavages in the fVIII heavy chain appear unaffected by the presence of vWF under these conditions. B domain cleavages obviously are fast relative to those at positions 372 and 1689 since the heavy chain bands that are larger than fVIII,..,A3 disappear rapidly. The relationship between cleavage events and the activation of fVIII was quantitated using scanning densitometry. Fig. 3 shows results from the experiment depicted in Fig. 2 in which the activation of fVIII was also determined. Percent cleavage at positions 372 and 1689 are shown along with activation kinetics. Cleavage at position 372 parallels the activation of fVIII in either the presence or absence of vWF. However, the rate of cleavage at position 1689 is increased in the presence of vWF such that near complete conversion has occurred prior to the development of peak activity. These data indicate that the rate enhancement of fVIII, cleavage at position 1689 due to vWF accounts for the observation that vWF does not inhibit fVIII activation, since the critical releasing cleavage becomes fast relative to cleavage at position 372.

In addition to bands corresponding to known fragments of fVIII, the 125I-labeled fVIII preparation shown in Fig. 2 contained an unidentified, thrombin-sensitive band (marked with an asterisk). This band constituted 7% of the radioactivity in the preparation as judged by SDS-PAGE and densitometric scanning. It was not present in the unlabeled fVIII preparation. It probably is a heavy-chain fragment since heavy-chain determinates govern the elution of fVIII heterodimers from Mono-Q. Repeating the experiment depicted in Fig. 2 in the absence of 125I-labeled fVIII, followed by silver staining gave the same overall qualitative results. Therefore, we do not consider the minor contaminant in the 125I-labeled fVIII preparation to influence the kinetic interpretations in this study.

Steady-state Kinetics of Bond Cleavages in FVIII and FVIII-vWF Complex by Thrombin—The kinetic parameters associated with three cleavage events in fVIII and fVIII-vWF complex by thrombin were done by fitting the velocity of bond cleavage to the nominal fVIII concentration using the Michaelis-Menten equation. The method of Lee and Wilson (19) was used to calculate average velocity as a function of average substrate concentration for the fitting procedure. The conversion of fVIII, to fVIII, (representing cleavage at position 1689), the formation of the fVIII, and fVIII, fragments (representing cleavage at position 372), and the disappearance of the bands migrating at 130 and 166 kDa (corresponding to cleavage at position 740 and elsewhere in the B domain) were measured densitometrically following SDS-PAGE as described under "Experimental Procedures." Fig. 4 shows fits of the data to the Michaelis-Menten equation using nonlinear least-squares regression analysis for the three cleavage reactions with the Eadie-Hofstee transformations included in the insets. In the top panel, the velocity of fVIII, cleavage is shown for both fVIII and fVIII-vWF complex. There is approximately an 8-fold increase in the catalytic efficiency (kcat/ Km) in the presence of vWF which is due to a decrease in the Km (Table I). Cleavage at position 740 within the B domain (bottom panel) responsible for the disappearance of high molecular weight forms of fVIII heavy chain is fast (note the difference in the y axis scale) relative to the subsequent cleavage at position 372. Because cleavage at position 740 is rapid, the rate of fVIII, and fVIII, formation is equivalent to the rate of cleavage at position 372.

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In addition to bands corresponding to known fragments of fVIII, the 125I-labeled fVIII preparation shown in Fig. 2 contained an unidentified, thrombin-sensitive band (marked with an asterisk). This band constituted 7% of the radioactivity in the preparation as judged by SDS-PAGE and densitometric scanning. It was not present in the unlabeled starting material. It represented approximately 10% of the starting material by scanning densitometry. HC, heavy chains; LC, light chains; A1-A2, fVIII,..,A3-A3-C1-C2; fVIII,..,A3-C1-C2, fVIII,..,A3-A1, fVIII,..,A2, fVIII,..,A2; df, dye front.

Steady-state Kinetics of Bond Cleavages in FVIII and FVIII-vWF Complex by Thrombin—The kinetic parameters associated with three cleavage events in fVIII and fVIII-vWF complex by thrombin were done by fitting the velocity of bond cleavage to the nominal fVIII concentration using the Michaelis-Menten equation. The method of Lee and Wilson (19) was used to calculate average velocity as a function of average substrate concentration for the fitting procedure. The conversion of fVIII, to fVIII, (representing cleavage at position 1689), the formation of the fVIII, and fVIII, fragments (representing cleavage at position 372), and the disappearance of the bands migrating at 130 and 166 kDa (corresponding to cleavage at position 740 and elsewhere in the B domain) were measured densitometrically following SDS-PAGE as described under "Experimental Procedures." Fig. 4 shows fits of the data to the Michaelis-Menten equation using nonlinear least-squares regression analysis for the three cleavage reactions with the Eadie-Hofstee transformations included in the insets. In the top panel, the velocity of fVIII, cleavage is shown for both fVIII and fVIII-vWF complex. There is approximately an 8-fold increase in the catalytic efficiency (kcat/ Km) in the presence of vWF which is due to a decrease in the Km (Table I). Cleavage at position 740 within the B domain (bottom panel) responsible for the disappearance of high molecular weight forms of fVIII heavy chain is fast (note the difference in the y axis scale) relative to the subsequent cleavage at position 372. Because cleavage at position 740 is rapid, the rate of fVIII, and fVIII, formation is equivalent to the rate of cleavage at position 372.
Activation of Factor VIII-von Willebrand Factor Complex by Thrombin

When larger concentrations of fVIII are used, fVIII heavy-chain cleavages, both within the B domain (bottom panel) and at position 372 (middle panel), are also modulated by the presence of vWF. Both $k_{\text{cat}}$ and $K_m$ values for these cleavages are greater in the absence of vWF, although the catalytic efficiency is unchanged (Table I). Since the catalytic efficiency reflects the velocity at substrate concentrations below $K_m$ and since the concentration of fVIII in human plasma is probably about 1 nM, the rates of heavy-chain cleavages are not influenced by vWF at physiological concentrations.

Effect of vWF on the Cleavage of Isolated fVIIIc by Thrombin—The kinetics of cleavage of $^{125}$I-labeled fVIIIc by thrombin were determined under single turnover conditions as described under “Experimental Procedures” to obtain the kinetic parameters $K_m$ and $k_{\text{cat}}$ of this reaction (Table I). As in the case of intact fVIII, vWF increases the catalytic efficiency of this reaction by decreasing the $K_m$. The $K_m$ values for cleavage of isolated fVIIIc and fVIIIc in intact fVIII are not significantly different. However, the $k_{\text{cat}}$ values in either the presence or absence of vWF are much lower for isolated fVIIIc. Thus, the catalytic efficiency of cleavage of isolated fVIIIc is lower. This did not appear to be due to radiolabeling artifacts or due to single turnover reaction conditions, since cleavage of unlabeled, isolated fVIIIc under conditions of substrate excess was clearly slower than cleavage of fVIIIc in intact fVIII as judged by SDS-PAGE and silver staining (data not shown).

DISCUSSION

The activation of factor VIII by thrombin is associated with several proteolytic cleavages: at positions 372, 740, and 1689, plus additional unidentified cleavages in the B domain. The functional significance of these cleavages, even the subunit composition of the active product(s), has been controversial (reviewed in Refs. 9 and 10), because of the complexity of the cleavage patterns and the fact that activated factor VIII is unstable under the usual conditions used in the activation reaction (4, 5, 9–11, 22–25). Previous work in our laboratory using porcine fVIII free of vWF has led to the development of the following kinetic scheme, where the numbers in parentheses refer to the apparent molecular masses (kDa) of the subunits:

We have found that the fVIII$_{A1/A2/A3-C1-C2}$ heterotrimer is

![Fig. 3. Comparison of activation and cleavage of fVIII by thrombin.](http://www.jbc.org/)

![Fig. 4. Steady-state kinetics of cleavages in fVIII catalyzed by thrombin.](http://www.jbc.org/)
one species of activated fVIII and can be isolated in stable form at pH 6.0 (10). Subsequently, we found that the fVIII_{A2/Lc} heterotrimer (intermediate I) is another species of activated fVIII. Intermediate I can be isolated in stable form by treatment of fVIII with an enzyme from the venom of Bothrops jararacussu, which does not cleave fVIII_{Lc} (5).

The purpose of the present investigation was to study the activation of fVIII-vWF complex by thrombin for possible effects of vWF on the rate and extent of activation. The activation of fVIII-vWF complex is presumably the relevant reaction in vivo where fVIII is completely complexed to vWF. Fig. 1 shows that association of fVIII with vWF does not affect the activation rate, activatability, or subsequent inactivation of fVIII. Additionally, novel intermediates are not formed in the presence of vWF (Fig. 2). However, the catalytic efficiency of light-chain cleavage is accelerated in the presence of vWF approximately 8-fold (Table I). vWF has no measurable effect on the rate of heavy-chain cleavages at low concentrations of fVIII (Fig. 2) although at higher concentrations of fVIII, an inhibitory effect of vWF due to a decrease in the k_{cat} at positions 3'72 and 740 is evident. In either the presence or absence of vWF, cleavage at position 740 is faster relative to those at positions 372 (to form intermediate I) and 1689 (to form intermediate II).

Thus, starting with the 166/76 heterodimer, addition of thrombin results in the rapid formation of the fVIII_{A2/Lc} heterodimer in the presence and absence of vWF. In the presence of vWF, cleavage at position 1689 is fast relative to cleavage at position 372, that so intermediate II is the dominant, if not sole, intermediate in the kinetic pathway. In the absence of vWF, the catalytic efficiencies of cleavages at positions 372 and 1689 are similar (Table I), indicating that the traffic is approximately equal through both pathways.

In summary, the rate-limiting step in the activation of fVIII-vWF complex by thrombin is cleavage at position 372. The cofactor effect of vWF on light chain cleavage eliminates cleavage at position 1689 as a partially rate-limiting step. Thus, an inhibitory effect of vWF, due to intermediate I retained in complex, on the kinetics of fVIII activation is not observed. Intermediate II is the dominant species on the pathway and dissociates rapidly from vWF relative to cleavage at 372, otherwise vWF would inhibit the kinetics of activation.

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