Partial Purification and Characterization of a Protease From Human Plasma Cleaving von Willebrand Factor to Fragments Produced by In Vivo Proteolysis

By Miha Furlan, Rodolfo Robles, and Bernhard Lammle

Proteolytic cleavage of von Willebrand factor (vWF) takes place in the circulating blood of healthy subjects and is increased in some patients with von Willebrand disease type 2A. The hemostatically active large vWF multimers are degraded to smaller less active forms. It has been suggested that the polypeptide subunit of vWF is cleaved at the peptide bond 842Tyr-843Met. We purified (=10,000-fold) from human plasma a vWF-degrading protease, using chelating Sepharose, hydrophobic interaction chromatography, and gel filtration. The enzyme was found to be virtually absent in the platelet lysates obtained by repeated freezing and thawing. The proteolytic activity was associated with a high molecular weight protein (=300 kDa) as judged by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. vWF was resistant against the protease in a neutral buffer at physiological ionic strength but became degraded at low salt concentration or in the presence of 1 mol/L urea. No degradation of human fibrinogen, bovine serum albumin, or calf skin collagen by the purified protease was noted under the same experimental conditions. Proteolytic activity showed a pH optimum at 8 to 9 and was strongly inhibited by chelating agents, whereas only slow inhibition was observed with N-ethylmaleimide. There was no inhibition by iodoacetamide, leupeptin, or serine protease inhibitors. The best peptideyl diazomethyl ketone inhibitor was Z-Phe-Phe-CHN₂. Activation by divalent metal ions was found to increase in the following order: Zn²⁺>Cu²⁺>Cd²⁺>Ni²⁺≈Co²⁺<Mn²⁺<Mg²⁺<Ca²⁺<Sr²⁺<Ba²⁺. The observed properties of the vWF-degrading enzyme differ from those of all other hitherto described proteases. Purified vWF was incubated with the protease, and the degraded material subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis after disulfide reduction. The size, amino acid composition, and amino terminal sequence of the reduced fragments confirmed that the peptide bond 842Tyr-843Met had been cleaved, ie, the same bond that has been proposed to be cleaved in vivo.

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VON WILLEBRAND FACTOR (vWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kDa. Multimeric forms of vWF are composed of 250-kD polypeptide subunits linked together by disulfide bridges. vWF mediates the initial platelet adhesion to the subendothelium of the damaged vessel wall at high shear rates. Only the largest multimers are hemostatically active. It is assumed that endothelial cells secrete large polymeric forms of vWF and that low molecular weight (LMW) forms of vWF arise from proteolytic cleavage.1 Proteolytic degradation of vWF is a normal occurrence in healthy individuals2 but may be increased in patients with von Willebrand disease (vWD) type 2A,2,6 causing the absence of large vWF multimers. A small but consistent proportion of vWF in normal plasma is composed of 189-, 176-, and 140-kD fragments resulting from proteolytic degradation of vWF in vivo; ie, the 140-kD fragment was derived from the N-terminal region, whereas the 176-kD fragment originates from the C-terminal region of the subunit. LMW forms of vWF were isolated from normal human plasma and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after disulfide reduction; the unusually high proportion of vWF fragments is compatible with the view that the LMW forms of vWF have been partially8 or predominantly9 derived from large multimers by proteolytic degradation. The infusion of 1-desamino-8-D-arginine vaspressin in normal subjects was followed by the appearance in plasma of very large multimers that were subsequently rapidly cleared because of proteolytic degradation.10 In acquired vWD associated with the myeloproliferative syndrome, the lack of large vWF multimers and an increase of proteolytic fragments were observed,11 indicating increased proteolysis in vivo. Unusually large molecular forms of vWF were found in patients with thrombotic thrombocytopenic purpura (TTP);12 these abnormally large multimers disappeared after transfusion of normal fresh frozen plasma.13 It is evident that proteolytic enzyme(s) may be involved in the physiological regulation of the polymeric size of vWF in the circulating blood and may also play an important role in the pathogenesis of vWF abnormalities in some patients with congenital and acquired disorders of hemostasis.

The proteolytic enzyme responsible for vWF degradation in normal human plasma has not yet been identified. Several proteases have been shown to cleave vWF, thereby impairing its binding affinity for platelets. Although plasmin cleaved several peptide bonds in vWF, there remained a high molecular weight (HMW) core retaining about 70% of the platelet-agglutinating activity determined as ristocetin cofactor.14 A 34-kD peptide was split from the N-termini of the individual vWF subunits in the early stage of digestion by plasmin.15 Epitope mapping of plasmin-induced fragments showed that they originated from regions of the vWF subunit different from those giving rise to the fragments present in circulating plasma.17 Porcine pancreatic elastase16 and serine proteases released from human leukocytes17,18 were shown to degrade

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4223
vWF with a resultant loss of large multimers. Epitope mapping of the degradation products indicated that human leukocyte elastase produced proteolytic fragments differing from those present in normal plasma and in vWD type 2A. Moreover, vWF multimeric patterns in plasma samples from patients with extremely high or low neutrophil counts were not significantly different from those in normal human plasma.

In addition to the above serine proteases a calpain-like protease released from human platelets was also shown to degrade large vWF multimers. Furthermore, analysis of the circulating vWF fragments indicated that the peptide bond between amino acid residues 842Tyr and 843Met, a site tentatively reflecting the specificity of calpains, was cleaved in the vWF subunit of patients with vWD type 2A. However, results of epitope mapping studies showed that calpains from porcine erythrocytes and porcine kidney failed to generate the vWF fragments produced in vivo.

In the present report, we attempted to purify from normal human plasma a protease degrading vWF to LMW forms and producing peptide fragments physiologically occurring in circulating blood. The results of our studies provide evidence that the proteolytic activity, cleaving the peptide bond 842Tyr-843Met in the vWF subunit, is associated with a HMW protein that is different from known serine proteases, cathepsins, matrix metalloproteinases, and calpains.

MATERIALS AND METHODS

Materials. EDTA, glycine, and urea were purchased from Merck (Darmstadt, Germany). Phenylmethylsulfonyl fluoride (PMSF), N-α-p-tosyl-L-lysine-chloromethylketone (TLCK), N-α-p-tosyl-L-phenylalanine-chloromethylketone (TPCK), leupeptin, EGTA, N-ethylmaleimide (NEM), iodoacetamide (IAA), dithiothreitol (DTT), SDS, acrylamide, bisacrylamide, dianinobenzidine, and Nonidet P-40 were from Fluka (Buchs, Switzerland). Disopropyl fluorophosphate (DFP) and bovine serum albumin (BSA) were from Sigma (St Louis, MO). Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, and HMW and LMW standards for SDS-electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Aprotinin was from Bayer (Wuppertal, Germany). D-phenylalanyl-prolyl-arginine-chloromethylketone (PPACK) was from Bachem (Bubendorf, Switzerland). Sepharose CL-2B, chelating Sepharose Fast Flow, butyl Sepharose 4 Fast Flow, and Sephacryl S-300 HR were purchased from Pharmacia-LKB (Uppsala, Sweden). Agarose Flow, butyl Sepharose 4 Fast Flow, and Sephacryl S-300 HR were used from Pharmacia-LKB. Peroxidase-labeled rabbit polyclonal rabbit antiserum against human vWF (RAHdFVIII) was kindly provided by Prof E. Shaw (Friedrich Miescher Institute, Basel, Switzerland). All other reagents were of analytical grade and were obtained from Fluka.

Purification of vWF. vWF was purified by gel filtration of human cryoprecipitate, obtained from 1,000 mL citrated plasma, on a 2.6-x 35-cm column of Sepharose CL-2B. Elution was performed with 0.13 mol/L NaCl/0.01 mol/L citrate/0.01 mol/L Tris-HCl, pH 7.4. Fractions of 6 mL were collected at a flow rate of 24 mL per hour. vWF antigen was measured by enzyme-linked immunosorbent assay.

Assay of the vWF cleaving activity. Because the degradation of vWF was found to be strongly affected by buffer conditions, the enzyme and the substrate were incubated on a circular dialysis membrane32 (Millipore VSWP; diameter, 25 mm) floating on the surface of 50 mL dialysis buffer contained in a 50-mL screw-cap plastic tube. In the search for optimum incubation conditions, the buffer composition was changed accordingly, but, in the subsequent studies, the dialysis solution was 1 mol/L urea in 5 mmol/L Tris-HCl buffer of pH 8.0. Full activation of the protease was achieved by preincubation for 5 minutes at 37°C with 10 mmol/L BaCl2. In a typical experiment, 50 μL activated protease solution and 100 μL substrate solution were carefully placed on the floating membrane, and the tube was closed and incubated for 24 hours in a dry oven at 37°C. Protease-free fractions of vWF from the Sepharose CL-2B column were used as substrate solution. The final vWF concentration in the incubation mixture was about 30 μg/mL. Reaction mixtures were then removed from the surface of the membrane filters and subjected to SDS-agarose gel electrophoresis to determine the multimeric pattern of vWF.

SDS-agarose gel electrophoresis and immunoblotting of unreduced vWF. Thin-layer agarose electrophoresis was performed using the discontinuous buffer system described by Rougier and Zimmerman. Before electrophoresis, each sample was incubated with an equal volume of the SDS-containing sample buffer for 15 minutes at 60°C. Horizontal electrophoresis in 1% HGT(P) agarose (2-mm thick, 20-cm wide, and 8.5-cm long) was performed for 17 hours at 16°C in an LKB Multiphor apparatus (Pharmacia-LKB) at 80 V and 10 mA. Proteins were electrotransferred to a nitrocellulose membrane for 3 hours at 26 V and 1.4 A using a Trans-Blot cell (Bio-Rad) in a buffer containing 0.04% SDS and 0.05 mol/L phosphate, pH 7.4. vWF multimers were identified with peroxidase-conjugated antibodies against human vWF (P226).

SDS-PAGE. SDS-5% polyacrylamide gels were prepared according to Laemmli. Proteins were reduced with 65 mmol/L DTT for 15 minutes at 60°C. Unreduced and reduced chromatographic fractions from the Sephacryl S-300 HR column were electrophoresed in 3-mm-thick gels for 18 hours at 60 V and were stained with Coomassie Blue.

Immunoblotting of reduced vWF. Reduced samples of un degraded and proteolytically cleaved vWF were subjected to SDS-PAGE and were electrotransferred to nitrocellulose as described above. For detection of vWF fragments, the nitrocellulose was incubated with rabbit antisera against human vWF (RAH/FVIII), followed by incubation with mouse antibodies against rabbit IgG (M737) and the APAAP reagent (K670) as described elsewhere.

Amino acid composition and sequence analysis. For analysis of amino acid composition and of amino acid sequence, the unreduced peak fraction of the protease and the reduced vWF fragments were dialyzed against 1 mmol/L EDTA, were lyophilized, and were dissolved in 1/50 original volume before SDS-PAGE. The HMW protein bands were electrotransferred from the gel to PVDF membrane for 6 hours at 26 V and 0.4 A, using 0.05% SDS/10% methanol/0.05 mol/L boric acid, pH 9.0, as transfer buffer. After transfer, the membrane protein was stained with Coomassie Blue in 40% methanol, destained with 40% methanol/10% acetic acid, and air-dried. The excised protein bands were transferred to the blot cartridge of the Applied Biosystems Model 477A Sequenator (Foster City, CA), which was equipped with on-line high-performance liquid chromatography for analysis of phenylthiohydroxantoin derivatives. For analysis of amino acid composition, the protein bands were hydrolyzed in the gas phase over 6 N HCl (for 22 hours at 110°C). Amino acids

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were extracted from the PVDF membrane with 70% 0.1 N HCl/30% methanol and were determined by high-performance liquid chromatography as phenylthiocarbamyl derivatives.

Preparation of blood components to be tested for the presence of the protease. Whole blood was drawn from a normal individual into a glass tube without anticoagulant. After 15 minutes at room temperature, the clotted blood sample was centrifuged twice for 15 minutes at 2,500g; 10 μL of 10 mM PPACK was added to 10 mL serum, and the sample was frozen at -20°C after incubation for 10 minutes at room temperature. From the same subject, 90 mL of blood was drawn into 10 mL of 0.13 mol/L NaCl-citrate in a plastic bottle. After centrifugation for 15 minutes at 300g, about 50 mL platelet-rich plasma (PRP) was recovered. One aliquot of PRP was centrifuged for 15 minutes at 2,500g. The resulting platelet-poor plasma (PPP) was reconstituted for 15 minutes at 3,000g and frozen at -20°C. The platelet sediment was resuspended in 0.9% NaCl (1/15 of the original volume) and was subjected to 3 cycles of freezing and thawing. Lyzed platelets were centrifuged for 15 minutes at 3,000g, and the resulting supernatant and sediment were frozen at -20°C. A frozen aliquot of PPP was slowly thawed to produce the cryoprecipitate. After centrifugation for 15 minutes at -5°C and 3,000g, the supernatant was added as cryoprecipitate, dissolved in 1/15 of the original volume of 0.9% NaCl, were frozen at -20°C. Another aliquot of PPP was transferred to a glass tube, mixed with 1/40 volume of 1mol/L CaCl2, and incubated for 15 minutes at 37°C. After removal of the fibrin clot and 15-minute centrifugation at 3,000g, PPACK (final concentration, 25 μmol/L) was added, and the fibrinogen was frozen at -20°C. Frozen samples were incubated for 10 minutes at 37°C before the assay of protease activity.

Purification of protease from plasma. Because the purification procedures required conditions that might induce activation of the coagulation cascade and clotting, fibrinogen was removed from plasma before chromatographic procedures were performed. In preliminary experiments, we noted that the activity of the vWF-degrading protease was not affected by defibrination, DFP, or PPACK. Blood (450 mL) from healthy volunteers was collected into citrate/dextrose/adenine (CPD-A) solution (63 mL) on a shaking balance. After 2 centrifugations for 15 minutes at 20°C and 2,500g, 1 mol/L CaCl2 was added to a final concentration of 25 mmol/L, and recalcified PPP was stirred for 30 minutes at 37°C. After removal of the fibrin clot by centrifugation, PPACK (final concentration, 5 μmol/L) and DFP (final concentration, 2 mmol/L) were added to the resulting serum and incubated for 15 minutes at 37°C to inhibit the activated clotting enzymes. Subsequently, the serum was dialyzed against the equilibrating buffer (1 mol/L NaCl/0.05 mol/L Tris-HCl, pH 7.4) required in the first chromatographic procedures. Aliquots of 50 mL were stored at -20°C until purification.

The protease was first purified on Cu2+-loaded chelating Sepharose (1.6 x 22 cm) using step-wise elution with equilibrating buffer containing increasing glycine concentration. Protease-containing fractions were pooled, dialyzed against 0.6 mol/L (NH4)2SO4/0.02 mol/L Tris-HCl, pH 7.0, and applied onto butyl Sepharose (1.6 x 27 cm). Most contaminating proteins were removed by step-wise elution at a lower (NH4)2SO4 concentration. Protolytically active fractions from two butyl Sepharose columns were pooled, dialyzed against 1 mmol/L EDTA, lyophilized, dissolved in 5 mL distilled water, and applied on a column (2.6 x 90 cm) of Sephacryl S-300 HR that had been equilibrated with 0.15 mol/L NaCl/0.01 mol/L Tris-HCl, pH 7.4. To improve the resolution of gel filtration, we simulated a long column by closing the first elution cycle (the proteins were once reintroduced into the same column) and by collecting the second cycle. Again, the active fractions were pooled, dialyzed against 1 mmol/L EDTA, lyophilized, dissolved in 3 mL distilled water, and resubmitted to gel filtration on the same Sephacryl S-300 HR column. This time the first two cycles were closed and the fractions of the third cycle were collected. In all the above chromatographic procedures, fractions of 6 mL were collected at a flow rate of 60 mL per hour. Fractions containing the protease were stored at -20°C.

Influence of metal ions and pH on the activity of the vWF-cleaving protease. Aliquots (95-μL) of the purified protease were incubated for 15 minutes at 37°C with 5-μL 0.2 mol/L solutions of the following salts: ZnCl2, CuSO4, Cd(CH3COO)2, CoSO4, NiCl2, MnCl2, MgCl2, CaCl2, SrCl2, and BaCl2. Then, 50 μL of the purified vWF was added to each aliquot, and the incubation mixtures were transferred onto floating membrane filters. After incubation for 24 hours at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl, pH 7.4, the samples were removed from the filters and applied to SDS-agarose electrophoresis. We determined pH optima of the protease after activation by Ca2+ and Ba2+. Protease was preincubated with 10 mmol/L CaCl2 or 10 mmol/L BaCl2 as described above and then was incubated with vWF while dialyzing against 1 mol/L urea/5 mmol/L Tris-HCl buffer adjusted to different pH values within the range of 6 to 11. The multimercular patterns of vWF in the dialyzed samples were analyzed by SDS-agarose electrophoresis.

Testing of protease inhibitors. Purified protease was preincubated for 5 minutes at 37°C with 10 mmol/L Ba2+ and then incubated for 15 minutes at 37°C with the following protease inhibitors (values in the parentheses denote the final inhibitor concentrations during preincubation): EDTA (10 mmol/L), EGTA (10 mmol/L), trisodium citrate (10 mmol/L), IAAs (10 mmol/L), NEM (10 mmol/L), DFP (1 mmol/L), PMSF (1 mmol/L), TLCK (1 mmol/L), TPCK (1 mmol/L), leupeptin (0.01 mmol/L), and apronin (0.01 mmol/L). In addition, the following carbobenzoxyl (Z) peptidyl-benzamidobenzylketone inhibitors were tested (all at a final concentration of 0.1 mmol/L during preincubation with the Ba2+-activated protease): Z-Leu-Leu-Tyr-CH2N2, Z-Val-Val-Tyr-CH2N2, Z-Phe-Ala-CH2N2, Z-Phe(1)-Ala-CH2N2, Z-Tyr-Ala-CH2N2, and Z-Phe-Phe-CH2N2. After preincubation with inhibitors, 100-μL aliquots of the enzyme/inhibitor mixtures were added to 50 μL solution of purified vWF, and the mixtures were dialyzed for 24 hours at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl, pH 7.4. Degradation of vWF was examined by SDS-agarose electrophoresis and immunoblotting.

Polypeptide subunits of degraded vWF and of other proteins. Purified vWF (50 μL) was mixed with different dilutions of the protease (100 μL), that had been preincubated with 10 mmol/L BaCl2 for 5 minutes at 37°C, and the mixtures were dialyzed for 24 hours at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl, pH 8.0. In addition, citrated normal human plasma (dilution 1:100) was dialyzed together with the protease. In control experiments, the protease was replaced by 0.15 mol/L NaCl/0.01 mol/L Tris-HCl, pH 7.4. After dialysis, the proteins were reduced with DTT and applied to SDS-PAGE. Coomassie-Blue staining was used for detection of polypeptide chains.

RESULTS

Detection of protease activity in PPP and in serum. A total of 10 μL of 10 mmol/L PPACK and 10 μL of 0.55 mmol/L CaCl2 were added to 200-μL samples of serum, PPP,
lysed platelets (x 15): sediment
lysed platelets (x 15): supernatant
platelet poor plasma (PPP)
cryoprecipitate free PPP
cryoprecipitate (x 15)
defibrinated PPP
serum

Fig 1. Degradation of purified vWF by normal human platelets, plasma, and serum. The amount of lysed platelets corresponded to a 15-fold volume of PRP and the amount of the cryoprecipitate to a 15-fold volume of PPP. Defibrinated PPP was obtained by recalcification of citrated PPP.

cryoprecipitate-free PPP, defibrinated PPP, as well as of 15-fold concentrated cryoprecipitate and lysed platelets. After incubation for 10 minutes at 37°C, 10-μL aliquots were mixed with 40 μL of vWF solution. After overnight dialysis at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl (pH 7.4), proteolytic degradation of vWF was assayed by SDS-agarose electrophoresis and immunoblotting. Protease activity was considerably higher in PPP than in the supernatant or the sediment of lysed platelets obtained from a 15-fold volume of PRP (Fig 1). We conclude that the enzyme does not originate from the platelets. The protease activity was not affected by defibrination and was partially recovered in the cryoprecipitate of PPP; the cryoprecipitate corresponding to a 15-fold amount of PPP showed considerable activity (Fig 1), but the nonconcentrated cryoprecipitate contained much less protease than did the equivalent amount of PPP (result not shown). There was no significant difference in the protease activity found in citrated PPP and in serum obtained from nonanticoagulated blood.

Separation of protease from vWF by gel filtration. vWF was purified by gel filtration on Sepharose CL-2B of human cryoprecipitate recovered from 1 L of citrated normal plasma (Fig 2). Fractions no. 12 to 20 contained vWF that was virtually devoid of contaminating proteins. These fractions were used as substrate in the assay of the vWF-degrading protease activity.

Degradation of vWF in gel filtration fractions by contaminating protease(s). Sepharose CL-2B fractions were subjected to dialysis against 0.13 mol/L NaCl/0.01 mol/L citrate/0.01 mol/L Tris-HCl, pH 7.4. SDS-agarose gel (Fig 3A) showed that vWF was extremely stable for 24 hours at 37°C at physiological salt concentration in the presence of 10 mmol/L citrate and no other inhibitors. Identical multimeric patterns were observed with the same Sepharose fractions before dialysis (results not shown). vWF in fractions no. 11 to 20 remained also virtually unchanged after dialysis for 24 hours at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl, pH 7.4 (Fig 3B). However, in the subsequent fractions, vWF was strongly degraded at low salt concentration in the presence of urea. It should be noted that the gel filtration fractions from the Sepharose CL-2B column had been collected in a buffer containing 10 mmol/L citrate and that no calcium ions were added to the dialysis buffer. Our results suggest that vWF is rendered susceptible to proteolytic degradation under denaturing conditions and that the proteolytic activity is not an integral property of the vWF molecule.

Effect of salt and urea on proteolytic cleavage of vWF. Aliquots of the Sepharose CL-2B fraction no. 29 were dialyzed against 5 mmol/L Tris-HCl (pH 7.4) containing different salt concentrations in the presence and absence of urea. No calcium ions were added. Parallel assays were performed with the fraction no. 29 that had been preincubated for 5 minutes at 37°C with 1 mol/L DFP before dialysis. Figure 4 shows disappearance of large vWF multimers after dialysis in the absence of NaCl. On the other hand, addition of 1 mol/L urea significantly enhanced vWF degradation even at physiological salt concentration. Combination of low salt concentration and 1 mol/L urea led to complete degradation of vWF. Our results also showed virtually identical proteolytic activity in the samples that had been preincubated with 1 mol/L DFP, a potent inhibitor of serine proteases. Based on these results, we chose the optimum conditions for a

Fig 2. Purification of vWF. (A) Gel filtration of cryoprecipitate on Sepharose CL-2B. (●), absorbance at 280 nm; (△), vWF-Antigen. (B) SDS-PAGE of gel filtration fractions (60 μL/lane) after disulfide reduction. Arrows denote migration distances of reduced fibronectin (FN) and of fibrinogen (Fbg) chains (Aα, Bβ, and γ).
sensitive assay of the protease activity, i.e., dialysis at 37°C against 1 mol/L urea/5 mmol/L Tris-HCl.

**Purification of the protease.** Defibrinated normal plasma (50 mL), prepared by recalcification of the citrated plasma as described in the Materials and Methods, was first fractionated by chelating chromatography (Fig 5A). Pooled fractions under the horizontal bar contained the vWF-degrading activity. The recovery of total protein in this pool was 14.7% of initial, as calculated from the product of pool, volume and absorbance units at 280 nm. Pooled material was further fractionated by hydrophobic chromatography on butyl Sepharose (Fig 5B). The pooled fractions under the bar contained virtually all protease activity, but only 0.75% of the initial protein. Butyl Sepharose fractions of two chromatographic separations were pooled, concentrated, and applied onto a 90-cm-long gel filtration column of Sephacryl S-300 HR (Fig 5C). To improve the resolution of the column, the eluted proteins of the first cycle were reintroduced into the column and collected in the second cycle. The protease activity was eluted between two HMW protein peaks. Because of considerable overlapping of these peaks, the fractions under the bar, containing all protease activity and 0.08% of initial total protein, were pooled and rechromatographed on the same...
Fig 6. Rechromatography of the protease on Sephacryl S-300 HR. Pooled fractions from the first Sephacryl S-300 HR fractionation shown in Fig 5C were concentrated and rechromatographed on the same column. The gel filtration fractions were twice recycled before collecting. (A) The elution diagram of proteins in the third gel filtration cycle is shown. (B) Protease activity in the collected fractions is shown. The immunoblot of vWF degradation was obtained after dialysis for 6 hours at 37°C of mixtures of purified vWF and individual Sephacryl fractions.

Figure 6 shows elution of the UV-absorbing material (Fig 6A) and of the protease activity (Fig 6B). Peak of the protease activity (fractions no. 9 to 17) contained only 0.009% of the initial serum protein. Thus, a purification factor of about 10,000 was achieved. Nevertheless, the final protease preparation still contained considerable amounts of contaminating proteins (Fig 7). The peak of the protease activity (fractions no. 11 to 15) coincided with the appearance in the unreduced gel of a protein band with a molecular weight (M_r) of about 300 kD, that was contaminated with several proteins having an M_r in the range between 130 and 450 kD (main bands with M_r of 450, 200, 180, and 130 kD). It should be noted that the protein bands of an M_r less than 80 kD in the unreduced gel reflect overlapping of the LMW proteins of the second cycle with elution of large proteins in the third cycle. After disulfide reduction, the strongest Coomassie staining was associated with polypeptide bands of 65 and 50 kD, and only a small fraction of total protein migrated with an M_r greater than 80 kD.

Fig 7. SDS-PAGE of unreduced (A) and reduced (B) fractions of the third elution cycle collected from Sephacryl S-300 HR (shown in Fig 6). The proteins were stained with Coomassie Blue. Arrows indicate migration distances of purified unreduced and reduced fibronectin (FN) and α2MG, as well as those of the commercially available molecular weight markers and their M_r (in kilodaltons [kD]). A total of 3 mL of each fraction was dialyzed against 1 mmol/L EDTA and was lyophilized, and the dry residue was dissolved in 100 µL of the SDS-containing sample buffer. Aliquots of 33 µL and 67-µL sample were subjected to unreduced and reduced SDS-PAGE, respectively. Thus, the material in each lane corresponds to 1 mL of unreduced or 2 mL of reduced Sephacryl fraction.

Activation by metal ions and pH optimum of the vWF cleaving protease. Purified protease was preincubated for 15 minutes at 37°C with various divalent ions (final concentration, 10 mmol/L). Their influence as potential protease activators was examined by dialysis of the mixture of the preincubated protease with vWF against 1 mol/L urea/5 mmol/L Tris-HCl (pH 7.4). The protease showed no activation by Zn²⁺, Cu²⁺, Cd²⁺, Co²⁺, Ni²⁺, or Mn²⁺; only slight activation by Mg²⁺; substantial activation by Ca²⁺ and Sr²⁺; and maximum activation by Ba²⁺ (Fig 8A). It should be noted that prolonged preincubation with the latter three divalent ions was associated with a loss of protease activity even at physiological salt concentration and in the absence of urea (results not shown), probably because of autodigestion of the protease. On the other hand, in the absence of these ions the protease was quite stable in solution; thus, it was possible to purify the enzyme by chromatographic procedures taking place for several days at room temperature.

pH optimum for vWF degradation by the Ca²⁺-activated protease was found at 9 to 10, whereas the highest activity of the Ba²⁺-activated protease was at pH 8 (Fig 8B). Based
on these results, subsequent assays of the protease activity were performed at pH 8 after the protease had been preincubated with 10 mmol/L BaCl₂.

Effect of protease inhibitors on vWF degradation. Immunoblots of SDS-agarose gels in Fig 9A showed that the cleavage agents EDTA and EGTA completely, whereas citrate only partially, inhibited vWF degradation by the Ba⁺²-preactivated protease. A 15-minute preincubation with sulfhydryl enzyme inhibitors IAA and NEM did not result in protease inhibition. There was also no inhibition by serine protease inhibitors DFP, PMSF and aprotinin, or by serine/sulfhydryl protease inhibitors TLCK, TPCK, and leupeptin. In other experiments (results not shown), the same inhibitors were incorporated also into the dialysis solution. Except for partial protease inhibition by NEM, all other multimeric vWF patterns were equal to those shown in Fig 9A. Thus, it appears that the protease is very slowly inhibited by NEM, albeit not by IAA. Among the peptidyl diazomethylketone inhibitors tested, only Z-Phe-Phe-CHN₂ and Z-Val-Val-Tyr-CHN₂ impaired the proteolytic degradation of vWF (Fig 9B).

Polypeptide subunits of degraded vWF and of other proteins. Incubation of vWF with the purified protease resulted in a concentration-dependent decrease of the intact vWF subunit that was accompanied by the appearance of
Fig 10. Polypeptide subunits of degraded vWF. Immunoblot of disulfide-reduced vWF and its fragments produced during incubation for 24 hours at 37°C with various dilutions of the Ba"1-activated protease in 1 mol/L urea/5 mmol/L Tris-HCl (pH 8.0). Control sample in the first lane was incubated under the same conditions without the enzyme. Arrows indicate the M_r values (in kilodaltons [kD]), estimated from the calibration curve obtained with molecular weight markers.

Increasing amounts of two fragments of M_r 170 and 140 kD as shown by immunoblots of reduced SDS-PAGE (Fig 10). Under conditions leading to complete cleavage of the M_r 250-kD vWF subunit into 170- and 140-kD fragments, there was no degradation of human fibrinogen, BSA or human serum albumin, or calf skin collagen (Fig 11); reduced SDS-PAGE showed unchanged subunit chains of these proteins used as potential substrates, indicating that the protease possesses a high specificity for vWF.

Amino acid analysis and amino acid sequence of vWF and its degradation products. All three electrophoretic bands (M_r 250, 170, and 140 kD) shown in Fig 10 were transferred onto PVDF membrane and subjected to analysis of amino acid composition and sequence. Results in Table 2 show good agreement between amino acid composition of these three polypeptide bands and the theoretical values calculated for the intact vWF subunit, C-terminal fragment 843-2050, and N-terminal fragment 1-842, respectively. N-terminal amino acid sequence of the 250- and 140-kD bands was Ser-Leu-Ser-X-Arg. This sequence agrees with that of the intact vWF subunit. Analysis of the larger degradation product (M_r 170 kD) yielded the sequence Met-Val-Thr-Gly-Asn corresponding to amino acid residues 843-847 in the intact vWF subunit. These data indicate that the purified protease cleaved the peptide bond 842Tyr-843Met.

DISCUSSION

The multimeric size is known to be one of the main determinants of the binding affinity for platelets and collagen, as well as of the hemostatic efficiency of vWF. Lack of large multimers is responsible for the bleeding tendency in vWD type 2A. On the other hand, abnormally large vWF multimers seem to be involved in the pathogenesis of intravascular platelet aggregation, thrombus formation, and thrombocytopenia in patients with chronic relapsing TTP. Supranormal vWF multimers have been shown to enhance platelet aggregation under conditions of high shear stress, such as that which might occur in the partially stenosed vessels of the microcirculation in patients with TTP.

It has been suggested that vWF is released from endothelial cells as a very large polymer that is converted in plasma to a series of smaller molecular forms. A small but consistent proportion of vWF subunits in normal plasma are degraded to fragments with apparent molecular masses of 189, 176,
Table 2. Amino Acid Composition of the Intact vWF Subunit (M. = 250 kD) and of Two Proteolytic Fragments (M. = 170 kD and M. = 140 kD)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>M. = 250 kD</th>
<th>vWF 1-2050</th>
<th>M. = 170 kD</th>
<th>vWF 843-2050</th>
<th>M. = 140 kD</th>
<th>vWF 1-842</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp + Asn</td>
<td>173</td>
<td>187</td>
<td>102</td>
<td>108</td>
<td>77</td>
<td>79</td>
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<tr>
<td>Thr</td>
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<td>116</td>
<td>70</td>
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</tr>
<tr>
<td>Ser</td>
<td>131</td>
<td>141</td>
<td>78</td>
<td>80</td>
<td>55</td>
<td>61</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>249</td>
<td>237</td>
<td>152</td>
<td>139</td>
<td>104</td>
<td>98</td>
</tr>
<tr>
<td>Pro</td>
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<td>136</td>
<td>85</td>
<td>86</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>Gly</td>
<td>152</td>
<td>137</td>
<td>117</td>
<td>88</td>
<td>76</td>
<td>51</td>
</tr>
<tr>
<td>Ala</td>
<td>110</td>
<td>104</td>
<td>67</td>
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<td>Val</td>
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<td>79</td>
<td>41</td>
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<td>30</td>
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<tr>
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<td>156</td>
<td>86</td>
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<td>73</td>
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<tr>
<td>Tyr</td>
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<td>49</td>
<td>18</td>
<td>22</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>Phe</td>
<td>58</td>
<td>56</td>
<td>33</td>
<td>32</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>His</td>
<td>53</td>
<td>52</td>
<td>28</td>
<td>31</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Lys</td>
<td>87</td>
<td>88</td>
<td>45</td>
<td>47</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Arg</td>
<td>95</td>
<td>101</td>
<td>49</td>
<td>58</td>
<td>38</td>
<td>43</td>
</tr>
</tbody>
</table>

Results are expressed as numbers of amino acid residues per theoretical number of residues (corrected for residues of Cys, Met and Trp that had not been determined by amino acid analysis) in the intact vWF subunit (1822 residues), C-terminal fragment (1057 residues), and N-terminal fragment (765 residues), respectively. Theoretical numbers of amino acid residues in the intact vWF subunit, as well as in the fragment 843-2050 and 1-842 are listed for comparison.

and 140 kD, indicating that proteolysis normally plays a role in the processing of vWF. Several proteases from plasma, granulocytes, or platelets have been shown to degrade large vWF multimers in vitro, but the generated proteolytic fragments were different from those found in the circulating blood. Although the protease responsible for the posttranslational changes in the multimeric pattern of vWF has not yet been identified, evidence has been presented that a specific protease-sensitive bond between residues 842Tyr and 843Met in the vWF subunit, a site that appears to reflect the specificity of calpains, is cleaved in vivo.

Contradictory reports regarding in vitro degradation of vWF have been published. Gralnick and coworkers observed restoration of large multimers in plasma of patients with vWD type 2A by inclusion of protease inhibitors EDTA, NEM, and leupeptin in the anticoagulant. This suggested that the absence of large multimers in vWD type 2A resulted from in vitro proteolysis. Battle et al were not able to restore large multimers in plasma when blood of vWD type 2A patients was collected in the presence of the same inhibitors and proposed that the absence of large multimers was not an in vitro phenomenon. Berkowitz et al noted that NEM, included in the anticoagulant, was more efficient in suppressing formation of proteolytic fragments of vWF than was EDTA or leupeptin, suggesting that a cysteine protease was responsible for degradation of normal vWF in vitro. Most other investigators, including ourselves (unpublished observations), noted no decrease in vWF multimeric size when normal plasma was incubated in vitro in the absence of protease inhibitors. In a recent report, Tsai et al presented evidence that vWF was proteolytically cleaved when normal plasma was perfused through long capillary tubing at high shear rate. This shear-dependent vWF degradation was not observed in the presence of EGTA. These investigators proposed that the shear stress may cause conformational changes in vWF, making the proteolytic cleavage site more accessible to proteases.

The stability of vWF in plasma, and, consequently, the lack of a sensitive protease assay, was the main initial obstacle hindering our search for the vWF-degrading plasma protease. The solution of this problem was based on early observations of van Mourik et al and Bouma et al who observed a decrease in the size and in the platelet agglutinating activity of vWF after dialysis against buffers of low ionic strength. Furthermore, we made use of our earlier experience showing that vWF and a protease, which is capable of degrading vWF, can be partially separated by gel filtration on Sepharose CL-2B. In the present study, we fractionated the cryoprecipitate of normal human plasma on Sepharose CL-2B and dialed the gel filtration fractions against buffers of different ionic strength in the absence and presence of urea. Our results showed that vWF is extremely stable in the physiological saline solution containing 0.01 mol/L citrate but may become readily degraded at low salt concentration and in the presence of 1 mol/L urea. Based on these observations, our protease assay consisted of dialysis of vWF with the protease against 1 mol/L urea/5 mmol/L Tris-HCl, using floating dialysis membranes as support for the incubation mixtures. It has been shown that proteolytic degradation of several other protein substrates is enhanced under denaturing conditions. It is conceivable that the denaturation (precipitation with organic solvents, salts, and polyethylene glycol and treatment with detergents and heating) leads to proteolytic cleavage of vWF in the process of large-scale production and virus inactivation of factor VIII/vWF concentrates and is responsible for the loss of the largest multimers and of the therapeutic efficacy in vWD.

It has been reported that fibronectin undergoes spontaneous proteolytic degradation and that latent fibronectinase, gelatinase, laminase, and collagenase activities are integral
Our results showed that in the early eluting Sepharose CL-2B fractions, vWF was stable even at low ionic strength and in 1 mol/L urea, whereas, in the later column fractions, vWF was readily cleaved because of the presence of a protease. We conclude that the observed protease is not an integral part of vWF.

It has been concluded from the amino acid sequence preceding the peptide bond cleaved in vivo that a calcium-dependent neutral protease (calpain) might represent the enzyme involved in physiological vWF degradation. Moreover, a calpain released from platelets by detergent or sonication was shown to be capable of degrading vWF. Our experiments showed that the normal PPP had a remarkably higher vWF cleaving activity than did the supernatant or the pellet from platelet lysates prepared from a 15-fold amount of PRP, indicating that the protease did not originate from the platelets.

Fibrinogen would interfere in the purification of the protease from plasma and in the assay procedures requiring metal ions. Because dephosphorylation did not affect the protease activity, the citrated plasma was recalcified to remove fibrinogen in the first step of the protease purification. The protease was further purified by combination of three chromatographic methods, i.e., chelate chromatography, hydrophobic chromatography, and gel filtration. Although the final product was purified about 10,000-fold, the protease was still contaminated by a number of nonidentified proteins. The elution of the proteolytic activity coincided with the appearance of a protein with an M, of 300 kD. On reduction of disulfide bridges, the main polypeptide bands migrated in SDS-PAGE, with apparent M, of 65 and 50 kD.

The protease was activated by preincubation with divalent ions in the following order: Ba2+ > Sr2+ > Ca2+ > Mg2+. It was inhibited by EDTA, EGTA, and citrate. It is obvious that the protease is a metalloenzyme, but it differs from other known metalloproteases in that barium instead of calcium ions are the most efficient activators. Furthermore, the protease is different from matrix metalloproteinases because it does not require zinc ions for full activity. The enzyme has a pH optimum at 8 to 10, suggesting that the protease does not belong to acid lysosomal cathepsins. The enzyme is no serine protease because it was not affected by any of the serine protease inhibitors used. Lack of inhibition after a 15-minute preincubation with NEM or IAA suggested that free SH-groups are not required for the proteolytic activity. There was only a partial inhibition of the protease during 24-hour incubation with NEM; it is difficult to judge whether this inhibition was caused by a slowly reacting SH-group in the protease or by a side-reaction of another amino acid with 10 mmol/L NEM. Leupeptin, TLCK, and TPCK, capable of inhibiting several serine and cysteine proteinases, had no effect on vWF degradation by the purified protease. No inhibition was observed in the presence of Z-Leu-Leu-Tyr-CHN2, the fastest peptidyl diazomethylketone inhibitor of calpains. On the other hand, Z-Phe-Phe-CHN2, a very weak calpain inhibitor impaired vWF cleavage. Thus, our protease behaved very differently from calpain with regard to sulfhydryl reagents and peptidyl diazomethylketone inhibitors.

Considering the unusually HMW of the vWF-cleaving protease, there is a possibility that the enzyme is complexed with a plasma protease inhibitor such as α2-macroglobulin (α2-MG). Varimbo et al.43 reported a high purity factor VIII/vWF concentrate to gel filtration on Sepharose 4B; the peak of vWF was followed by a peak of amidase activity. The latter activity was associated with α2-MG but showed no proteolytic activity against azocasein as substrate. Although it is generally accepted that proteases in complex with α2-MG lose their ability to degrade protein substrates, it has been shown44 that thrombin-α2-MG-complex retains sufficient proteolytic activity to activate the procoagulant activity of factor VIII. Thus, the possibility cannot be ruled out that the cleavable bond in the denatured vWF may be attacked by the protease-α2-MG-complex.

Several lines of evidence argue against a circulating complex between α2-MG and the vWF-cleaving enzyme. Such complexes are usually recognized by high-affinity receptors on several cell types and are destined to rapid clearance.45 The α2-MG-bound protease should become readily inactivated by small synthetic inhibitors of serine or cysteine proteases. Migration of the protease band in the reduced SDS-PAGE (M, = 300 kD) was faster than that of α2-MG-dimer (M, = 360 kD), and there was no characteristic protein band noted in the reduced SDS-PAGE corresponding to the α2-MG-monomer (M, = 180 kD). Furthermore, the amino acid composition of the protein band associated with the protease activity was quite different from the amino acid composition of α2-MG.46 We performed the PROPSSEARCH database query, available on the Internet (http://www.embl-heidelberg.de/aaa.html), using the amino acid analysis data of the unreduced 300-kD protein band (Table 1). We were surprised to find the human vWF precursor on the top of the list of mammalian proteins with the highest score, whereas none of the plasma protease inhibitors was ranked within the best 200 hits. We performed an immunoblotting experiment in which the protease-containing fractions from the Sephacryl column were tested with a rabbit antiserum against vWF. None of them contained any detectable traces of vWF-related antigen (results not shown). Further studies are required to elucidate the identity of the plasma protease involved in degradation of vWF.

vWF, fibrinogen, BSA, calf skin collagen, and diluted human plasma were incubated with the purified protease. Although the vWF subunit was completely cleaved to fragments of 140 kD and 170 kD, we observed no degradation of the other potential protein substrates. Even the C-terminal free-floating tail of the fibrinogen αα-chain, known to be highly sensitive to degradation by most proteolytic enzymes, was completely retained after 24-hour incubation with the undiluted protease fraction. This suggests that the protease possesses a high specificity for vWF.

Amino acid compositions of the intact vWF subunit and of both fragments, generated during incubation of pure vWF with the protease and separated by SDS-PAGE, were compared with theoretical values for the intact vWF subunit and for its degradation products 1-842 and 843-2050. Our results showed good agreement between amino acid compositions of the bands of 140 kD, 170 kD, and 250 kD and the calculated values for vWF 1-842, vWF 843-2050, and vWF 1-
VON WILLEBRAND FACTOR-CLEAVING PROTEASE

2050, respectively. Moreover, N-terminal amino acid sequences of the 250-kD and 140-kD bands (Ser-Leu-Ser-X-Arg) were compatible with those of the intact mature vWF subunit, whereas the 170-kD band yielded the sequence Met-Val-Thr-Gly-Asn corresponding to amino acid residues vWF 843-847. In conclusion, our results show that the partially purified protease cleaves the peptide bond 842Tyr-843MetVal-Thr-Gly-Asn corresponding to amino acid residues 2050, respectively. Moreover, N-terminal amino acid sequence that is assumed to be split in vivo. It is conceivable that the protease described in this communication may be involved in the regulation of the polymeric size of vWF in the circulating blood and, thus, may affect primary hemostasis.

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Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis

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