A. G., & Paul, C., Eds.) pp 193–203, Editions de l'Universite de Bruxelles, Bruxelles.
Hofrichter, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., & Eaton, W. A. (1985) *Biochemistry 24*, 2667–2679.
Lindqvist, L., El Mohsni, S., Tfibel, F., & Alpert, B. (1980) *Nature (London) 288*, 729–730.
Lindqvist, L., El Mohsni, S., Tfibel, F., Alpert, B., & Andre, J. C. (1981) *Chem. Phys. Lett. 79*, 525–528.
Perutz, M. F., & Matthews, F. S. (1966) *J. Mol. Biol. 21*, 199–202.
Phillips, S. E. V., Hall, D., & Perutz, M. F. (1981) *J. Mol. Biol. 150*, 137–141.
Sorensen, L. B. (1980) *Dissertation*, University of Illinois—Urbana, Champaign, IL.
Stetzkowski, F., Banerjee, R., Marden, M. C., Beece, D. K., Bowne, S. F., Doster, W., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., & Jung, C. (1985) *J. Biol. Chem. 260*, 8803–8809.
Tucker, P. W., Philips, S. E. V., Perutz, M. F., Houtchens, R., & Caughey, W. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1076–1080.
Young, R. D. (1984) *J. Chem. Phys. 80*, 554–560.
Young, R. D., & Bowne, S. F. (1984) *J. Chem. Phys. 81*, 3730–3737.

Human von Willebrand Factor: A Multivalent Protein Composed of Identical Subunits†

Michael W. Chopek,* Jean-Pierre Girma,‡ Kazuo Fujikawa, Earl W. Davie, and Koiti Titani
Department of Biochemistry, University of Washington, Seattle, Washington 98195
Received August 27, 1985; Revised Manuscript Received March 4, 1986

ABSTRACT: A large-scale method for the isolation of von Willebrand factor (vWF) from human factor VIII concentrates was developed in order to study the structure of this protein and its platelet binding activity. vWF is composed of a number of glycoprotein subunits that are linked together by disulfide bonds to form a series of multimers. These multimers appear to contain an even number of subunits of 270K. Two minor components of M_r 140K and 120K were also identified, but these chains appear to result from minor proteolysis. The smallest multimer of vWF contained nearly equimolar amounts of the 270K, 140K, and 120K subunits, while the largest multimers contained less than 20% of the two minor components. Amino acid sequence analysis, amino acid composition, and cleavage by cyanogen bromide indicate that the 270K subunits are identical and each is a single polypeptide chain with an amino-terminal sequence of Ser-Leu-Ser-Cys-Arg-Pro-Pro-Met-Val-Lys and a carboxyl-terminal sequence of Glu-Cys-Lys-Cys-Ser-Pro-Arg-Lys-Cys-Ser-Lys. Platelet binding in the presence of ristocetin was 8-fold greater with multimers larger than five (i.e., containing more than 10 subunits of 270K) as compared to multimers less than three (containing less than six subunits of 270K). However, partially reduced vWF (M_r 500K), regardless of whether it was prepared from large or small molecular weight multimers, gave platelet binding similar to that of the smallest multimers. Likewise, partial proteolysis by elastase, thermolysin, trypsin, or chymotrypsin produced small "multimer-like" proteins with platelet binding properties similar to either partially reduced vWF or to the smallest multimers. We conclude that human vWF contains identical 270K subunits assembled into a multivalent structure. Disassembly by either partial reduction or partial proteolysis produces essentially monovalent protein with platelet binding properties similar to that of the smallest multimers. Multivalency is likely the primary factor responsible for the increase in biological activity with multimer size.

Human von Willebrand factor (vWF)[1] is a large glycoprotein that circulates in plasma as a series of subunits or multimers linked by disulfide bonds. A very small amount of vWF is also complexed with factor VIII, a trace protein absent or modified in hemophilia A (Hoyer, 1981). vWF is also involved in platelet adhesion to subendothelium, leading to the formation of the platelet plug during vascular damage (Tschopp et al., 1974; Meyer & Baumgartner, 1983). Accordingly, individuals lacking vWF may have low factor VIII coagulant activity resulting in impaired fibrin formation as well as prolonged bleeding time due to poor platelet plug formation. Biological activity of human vWF is measured in vitro by its ability to promote platelet aggregation or to bind to specific receptors on the platelet membrane (Kao et al., 1979). Both measurements require the presence of a cofactor, such as ristocetin (Harrison & McKee, 1983). Thrombin,

† This work was supported in part by National Institutes of Health Grant HL 16919 to E.W.D., Grant HL 29595 to K.T., and Training Grant T32 HL 07150. M.W.C. was the recipient of Individual Postdoctoral Fellowship HL 06281. J.-P.G. was the recipient of a Bourse d'étude des Laboratoires Anphar-Rolland and Grants from the Philippe Foundation, Inc., and NATO.
* Address correspondence to this author at St. Paul Regional Red Cross, St. Paul, MN 55107, or Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.
‡ Present address: INSERM U.143, Hôpital de Bicêtre, 94270 Le Kremlin-Bicêtre, France.

[1] Abbreviations: vWF, von Willebrand factor; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; V_e/V_t, elution volume at the protein peak (V_e) divided by the total volume of packed column bed (V_t).

0006-2960/86/0425-3146$01.50/0

adenosine diphosphate, arachidonic acid, collagen, and thromboxane A2 also induce vWF binding to platelets, but to a lesser extent. These latter components, however, may be important in platelet aggregation under physiological conditions (Fujimoto et al., 1982; Ruggeri et al., 1982; Di Minno et al., 1983; Pietu et al., 1984).

vWF appears as a series of high molecular weight multimers upon large-pore gel electrophoresis (Ruggeri & Zimmerman, 1980; Hoyer & Shainoff, 1980; Meyer et al., 1980). The exact molecular weight of the various multimers and the difference from one to the other remain controversial. The value observed for the smallest form of vWF varies from 0.5×10^6 to 1.2×10^6 according to the markers and the composition of the electrophoretic gel used for the determination (Counts et al., 1978; Perret et al., 1979; Meyer et al., 1980; Ruggeri & Zimmerman, 1980; Sixma et al., 1984). There is general agreement that vWF multimers are assembled by the addition of an integral number of protomers, which are identical with or very similar to the smallest multimer. Since the fully reduced subunit has a molecular weight of about 260K (Legaz et al., 1973), the protomer is thought to be either a dimer (Counts et al., 1978; Perret et al., 1979) or a tetramer (Ruggeri & Zimmerman, 1980; Hoyer & Shainoff, 1980; Meyer et al., 1980). The interchain disulfide bonds that form a dimer are quite stable, although mild reduction of a small number of sensitive disulfide bonds (Ohmori et al., 1982) causes stepwise disassembly of the vWF multimers (Counts et al., 1978). Similar changes in vWF multimers have been observed after modification or removal of penultimate galactose (Gralnick et al., 1983) or after sonication (Casillas et al., 1980; Chan & Chan, 1982).

Biological activity of vWF has been shown to be related to the size of the multimers. Analysis of vWF in plasma from patients lacking vWF activity (Meyer et al., 1980) and the failure to correct the bleeding time of such patients by preparations deficient in larger multimers (Weinstein & Deykin, 1979) has suggested that the biological activity is primarily associated with the larger multimers. This has been confirmed in vitro by measuring the ristocetin cofactor activity of vWF multimers partially separated by gel filtration (Martin et al., 1981). In addition, both mild reduction and modification of the penultimate galactose of vWF resulted in the formation of smaller multimer-like molecules with decreased platelet binding and ristocetin cofactor activity (Counts et al., 1978; Gralnick et al., 1981, 1983). However, Sixma et al. (1984) have recently reported that low molecular weight multimers prepared by gel filtration or limited reduction of purified vWF support platelet adhesion to the subendothelium similar to that of the high molecular weight forms.

To date, few studies have been undertaken to isolate and characterize the portion(s) of the vWF molecule that interact(s) with specific receptors on the platelet membrane. Prolonged digestion of native vWF does not lead to complete loss of ristocetin cofactor activity, even though no trace of intact vWF subunit could be detected by gel electrophoresis (Atichartakarn et al., 1978; Martin et al., 1980, 1981). A tryptic fragment of unreduced M_r 116K accounted for this residual activity.

The reason for the stronger interaction of large vWF multimers with platelets has not been adequately explained. In this investigation, the electrophoretic pattern, amino acid and carbohydrate composition, number of unique cyanogen bromide fragments, and terminal sequences of human vWF have been studied. These experiments show that vWF is composed of identical subunits of 270K, in addition to some smaller fragments resulting from proteolytic degradation. We have also compared the ristocetin-induced platelet binding of purified vWF with vWF subjected to partial reduction or partial proteolysis. A decrease in vWF size by either partial proteolysis or partial reduction produces a protein with platelet binding properties similar to that of the smallest multimers. This suggests that vWF is multivalent and that multivalency is the primary reason for the variation in biological activity.

Experimental Procedures

Materials. The following were purchased from the suppliers indicated: Sepharose, Sephadex, and low molecular weight standards (Pharmacia Fine Chemicals, Piscataway, NJ); agarose [Sea-Kem HGT(P)] and Gelbond (Marine Colloid, Rockland, ME); Kodak-X-Omat AR film and cyanogen bromide (Eastman Kodak Co., Rochester, NY); Iodo-gen (Pierce Chemical Co., Rockford, IL); ristocetin sulfate (H. Lundbeck Co., Copenhagen, Denmark); Nonidet P-40 (British Drug House, Poole, England); horseradish peroxidase conjugated goat anti-rabbit IgG, gelatin (swine skin type I), phenylmethanesulfonyl flouride, and bovine serum albumin (Sigma Chemical Co., St. Louis, MO); agmatine sulfate, benzamidine, and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (water-soluble carbodiimide) (Aldrich Chemical Co., Milwaukee, WI); polybrene (Pierce Chemical Co.); [^{3}H]iodoacetic acid (New England Nuclear); alkaline phosphatase and goat anti-rabbit IgG (Miles Laboratories Inc., Elkhart, IN); rabbit anti-vWF antibody and human fibrinogen (Calbiochem-Behring Corp., La Jolla, CA); rabbit anti-human fibronectin antibody (Cappel Laboratories, Cochranville, PA). Rabbit myosin and plasma from a patient with IgM myeloma were kindly provided by Drs. E. Fischer and B. Gilliland, University of Washington, respectively.

Purification of Human vWF. Human vWF was purified from a commercial factor VIII concentrate generously provided by Dr. Henry Kingdon of Hyland Therapeutics, Division of Travenol Laboratories. The liquid concentrate containing 23 mg/mL protein and 16 units/mL factor VIII coagulant activity was made 0.3 M β-alanine, 10 mM benzamidine, 1 mM *o*-phenanthroline, 40 mg/L soybean trypsin inhibitor, and 0.1 mM diisopropyl fluorophosphate and then refrozen in 750-mL aliquots. Each aliquot was gel filtered at 450 mL/h in a siliconized 10×92 cm column of Sepharose CL-4B equilibrated with 150 mM NaCl, 20 mM imidazole hydrochloride pH 6.8, and 0.02% NaN_3 (imidazole buffer). This and all subsequent steps were performed at 22 °C unless otherwise noted. The early fractions of appropriate absorbance were pooled, and most contaminants were removed by passing this pool at 400–500 mL/h over two sequential plastic columns (6×12 cm) containing 350 mL of gelatin–Sepharose and 250 mL of agmatine–Sepharose. The columns were regenerated by washing with 500 mL of 8 M urea and 0.1 M sodium citrate, pH 5.5, followed by 1 L of distilled water and 1–2 L of imidazole buffer. Poly(ethylene glycol) 4000 was added to the fractions containing vWF (final concentration 12%), and the precipitate was collected by centrifugation. It was redissolved in a minimal volume (about 50 mL) of 0.5 M $CaCl_2$, 0.01 M NaCl, 0.05 M Tris-HCl, pH 7.4, and 0.02% NaN_3 (high calcium buffer) and made 5 mM in benzamidine. After centrifugation at 10000*g* for 10 min, it was subjected to gel filtration on a 5×91 cm siliconized column of Sepharose CL-4B equilibrated in the high calcium buffer. The fractions containing factor VIII coagulant activity were kept separate for further investigations. The fractions containing vWF were rapidly decalcified on Sephadex G-25 and precipitated in the presence of 40% $(NH_4)_2SO_4$ at 4 °C. The precipitate was

collected by centrifugation and stored as an $(NH_4)_2SO_4$ paste at −70 °C.

Protein Analysis. Protein was reduced with dithiothreitol and carboxymethylated with [^{3}H]iodoacetic acid by a modification of the method of Crestfield et al. (1963). S-Carboxymethylated (S-CM) protein was recovered by dialysis against distilled water. Five milligrams of S-CM protein was cleaved with 10 mg of cyanogen bromide in 72% formic acid at room temperature for 15 h (Gross & Witkop, 1962). The digest was lyophilized after 10-fold dilution with distilled water.

Separation of cyanogen bromide fragments was achieved by high-performance liquid chromatography (HPLC) on a Varian 5000 liquid chromatograph. The primary separation was achieved on three UltroPak TSK SW columns (LKB) connected in series, employing 6 M guanidine hydrochloride and 10 mM sodium phosphate, pH 6.0. Subsequent purification of fractions from size-exclusion HPLC was achieved by reverse-phase HPLC on either a Syn-Chropak RP-P column (SynChrom) or an Altex Ultrapore RPSC column (Beckman) with 0.1% trifluoroacetic acid in water as solvent A and 0.08% trifluoroacetic acid in acetonitrile as solvent B (Mahoney & Hermodson, 1980).

Amino acid compositions were determined on a Dionex amino acid analyzer (Model D-500) after 24, 48, and 96-h acid hydrolysis of S-CM protein. Tryptophan, neutral sugar, hexosamine, and neuraminic acid were determined as previously described (Fujikawa et al., 1977).

Automated sequence analyses were performed on a Beckman sequencer (Model 890C) in the presence of polybrene by the method of Edman and Begg (1967) with the program of Brauer et al. (1975). Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC in two complementary systems (Bridgen et al., 1976; Ericsson et al., 1977).

Protein concentration was estimated from absorbance at 280 nm with $E^{1\%}_{280}$ = 10.0 and corrected for light scattering by multiplying the absorbance at 320 nm by 1.7 (Cantor & Schimmel, 1980). The net protein absorbance at 280 nm was the measured absorbance at 280 nm minus 1.7 times the absorbance at 320 nm.

Preparation of Agmatine–Sepharose and Gelatin–Sepharose. Sepharose CL-4B (600 mL) activated with 200 g of CNBr (Cuatrescasas, 1970) was coupled to 100 g of ε-aminocaproic acid. Agmatine sulfate (10 g) was coupled to ε-aminocaproic acid–Sepharose at pH 4.8 overnight with 50 g of water-soluble carbodiimide. Sepharose CL-4B (500 mL) activated with 100 g of CNBr (March et al., 1974) was coupled to 5 g of gelatin at 0 °C. Both gels were washed and stored at 4 °C in a neutral saline buffer with 0.02% NaN_3.

Gel Electrophoresis and Western Blots. Vertical $NaDodSO_4$–urea acrylamide agarose slab gels (composite gels) were prepared according to the methods of Peacock and Dingman (1968) and Weber and Osborn (1969). $NaDodSO_4$ electrophoresis in polyacrylamide slab gels was performed by the method of Laemmli (1970) or the method of O'Farrell (1975). Two-dimensional gel electrophoresis was a combination of Laemmli and O'Farrell gels (composite gels). Western blots of composite or Laemmli gels were performed according to the method of Towbin et al. (1979). Staining was performed with either rabbit monospecific anti-vWF, monoclonal mouse anti-vWF, or anti-fibronectin as the first antibody and horseradish peroxidase conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG as the second antibody.

Quantitative Electrophoresis of vWF. Quantitative electrophoresis of vWF was performed in three systems: composite gels without reduction, Laemmli gels after reduction, and composite gels for isolation of individual multimers followed by Laemmli gels after reduction. Composite gels were either sliced into 1.2-mm pieces for counting or dried, stained, and autoradiographed. Stained gels and autoradiographs were scanned on a Joyce Loebel dual-beam microdensitometer. Laemmli gels were stained and scanned before drying. Stained bands from dried composite and Laemmli gels were also cut out and counted. Composition of isolated ^{125}I-labeled multimers was determined as follows: a 17 cm long gel was cut into 1 cm wide strips and then into 140 slices of 1.2 mm. Fractions were reduced and reelectrophoresed on a 7.5% Laemmli gel, and after being stained, dried, and autoradiographed, the bands were cut out, and the radioactivity was counted. Results were expressed as the percent that each band contributed to the total for that starting multimer. In order to judge purity and stability, unreduced gel slices from the first composite gel were reelectrophoresed on a second composite gel in parallel with control vWF. All steps of quantitative electrophoresis were performed in duplicate. The autoradiograph and Coomassie blue staining always coincided precisely for both composite and Laemmli gels. Calculations of number-, weight- and *Z*-average molecular weights were performed according to Cantor and Schimmel (1980).

Reduction. Partially reduced vWF was prepared by incubating the protein with 10 mM β-mercaptoethanol at pH 8.5, 22 °C, for 30 min followed by alkylation with 20 mM iodoacetamide or *N*-ethylmaleimide. Excess reagents were removed by gel filtration on Sephadex G-25 or by dialysis.

Platelet Binding in the Presence of Ristocetin. ^{125}I-labeled protein (0.5–0.9 μCi/μg, 0.1 μg/mL final) was mixed with 200 μL of formaldehyde-fixed platelets (10^8 cells/mL final) in 0.15 M NaCl and 0.025 M Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin, 0.1% Nonidet P-40, 0.02% NaN_3, and 2 mM phenylmethanesulfonyl fluoride. Ristocetin dissolved in the above buffer without bovine serum albumin was added in duplicates to a final concentration of 1.2 mg/mL. After 1 h at 22 °C without stirring, two aliquots (100 μL) of each platelet suspension were layered onto 250 μL of 20% sucrose in the same buffer in 400 μL conical polyethylene tubes and centrifuged at 11000 *g* for 5 min. Nonspecific binding was calculated as counts in the presence of ristocetin without platelets plus counts in the presence of platelets without ristocetin. These values were similar to the apparent binding observed in the presence of unlabeled vWF at the final concentration of 2.5 mg/mL. Platelet-bound protein was dissolved by suspending the pellet in 6 M urea and 2 mM phenylmethanesulfonyl fluoride, for 30 min at 40 °C. After centrifugation, the supernatant usually contained 70–80% of the radioactivity.

Effect of Multimer Size on Platelet Binding. Differences in platelet binding as a function of vWF multimer size were investigated by two methods. First, ^{125}I-labeled native vWF was allowed to react with platelets in the presence of ristocetin. The relative binding of competing vWF multimers was estimated by quantifying each individual multimer in both platelet-bound and platelet supernatant fractions with composite gel electrophoresis. Second, ^{125}I-vWF was partially resolved into different multimers on Sepharose CL-2B. A sample of 200 μL of ^{125}I-vWF (60 μg/mL) was gel filtered through a column of Sepharose CL-2B (0.7 × 50 cm) equilibrated in 0.15 NaCl and 0.025 M Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin. Fractions were dialyzed for 4 h at 22 °C against 0.15 M NaCl and 0.2 M Tris-HCl, pH 8.5, partially reduced, alkylated with *N*-

ethylmaleimide, and dialyzed against 0.15 M NaCl and 0.025 M Tris-HCl, pH 7.4, overnight at 4 °C. These samples were analyzed by composite gel electrophoresis and by platelet binding assay at final concentrations of ^{125}I-labeled protein between 0.05 and 0.15 μg/mL.

Proteolysis. Native vWF was digested with factor IXa, factor XIIa, thrombin, factor Xa, mast cell protease, *Staphylococcus aureus* V-8 protease, elastase, α-chymotrypsin, thermolysin, subtilisin, and trypsin. In a typical experiment, vWF at 1 mg/mL in 0.1 M NaCl and 0.05 M Tris-HCl, pH 7.8–8.0, was incubated at a 1:50 w/w enzyme/substrate ratio for 2 or 20 h at 22 °C. The reaction was stopped by adding 1/20 volume of freshly diluted 0.1 M diisopropyl fluorophosphate and incubating for at least 30 min at 22 °C. Thermolysin digestion was stopped by adding 10 mM EDTA. The digests were analyzed by gel electrophoresis both with and without reduction. Partially reduced vWF was occasionally substituted for native vWF. In separate experiments, ^{125}I-labeled vWF was digested at about 50 μg/mL with an enzyme/substrate ratio of 1:50.

Other Methods. Pooled normal human plasma stored at −70 °C was used as control for the vWF antigen assays, factor VIII coagulant activity, and ristocetin cofactor activity and was defined as containing 1.0 unit/mL for each activity. Routine measurement of vWF antigen was performed with a competitive enzyme-linked immunoassay (Engvall, 1980). Diluted samples were incubated overnight at 22 °C with 1:1100 rabbit anti-vWF antiserum and then allowed to react for 30 min at 22 °C with vWF-coated plates. Bound rabbit IgG was quantified with an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (1:1000, 2 h, 22 °C), *p*-nitrophenyl phosphate as substrate, and a Dynatech MR580 automatic plate reader. Immunoradiometric assay of vWF was performed by a two-site method with a specific immunopurified ^{125}I-labeled rabbit antibody (Ardaillou et al., 1978). Rocket immunoassays were kindly performed in the laboratory of Dr. R. Edson, University of Minnesota, by a modification of the method of Laurell (1966). Factor VIII coagulant activity was measured by a standard kaolin–cephalin clotting time (Proctor & Rapaport, 1961) with factor VIII deficient plasma and human brain cephalin (Hjort et al., 1955). Ristocetin cofactor activity was kindly determined in the laboratory of Dr. R. Counts, Puget Sound Blood Center, by a modification of the macroscopic aggregation test (Brinkhous et al., 1975). Outdated human platelets were fixed with formaldehyde by a modification of the method of Allain et al. (1975). Proteins were labeled with Na^{125}I by using Iodo-gen according to the method of Fraker and Speck (1978). Cross-linked fibrinogen was prepared as follows: a 20% solution in 1 M NaCl and 50 mM Tris-HCl, pH 7.4, was incubated for 10 min with 0.02 M glutaraldehyde and then for an additional 10 min with 0.2 M glycine. Samples were then diluted with 6 M urea, 1% $NaDodSO_4$, and 0.1 M Tris–H_3PO_4, pH 7.0, and stored at −70 °C. Human fibronectin was prepared from plasma or factor VIII concentrate by adsorption onto gelatin–Sepharose, washing with 1 M NaCl, and elution with 3 M urea and 0.05 M sodium citrate, pH 5.5.

Results

Purification of Human vWF. The present method for the purification of large amounts of vWF involves gel filtration on Sepharose CL-4B, removal of contaminants by gelatin–Sepharose and agmatine–Sepharose, and gel filtration on a second Sepharose CL-4B column in the presence of 0.5 M $CaCl_2$. vWF and factor VIII coagulant activity coeluted from the first Sepharose CL-4B column in a broad peak from 0.3 to beyond 0.7 V_e/V_t with a peak of 0.44. The early eluting fractions were pooled and contained 73% of the original vWF (Table I) and 79% of factor VIII coagulant activity. No special conditions were necessary for the quantitative adsorption of fibronectin by gelatin–Sepharose. Agmatine–Sepharose, however, was found to bind all proteins if they were applied to the column at low ionic strength. The various proteins were eluted with a neutral salt gradient in the order of vWF, fibronectin, and, finally, fibrinogen. Fibronectin and most of the fibrinogen were selectively removed by passing the preparation through the agmatine–Sepharose column at 0.15–0.3 M NaCl. With three different large batches of resin, the optimal ionic strength for sample application and elution was 0.15 M NaCl in two cases and 0.3 M NaCl in the third. Step yields were 90% or greater for both vWF and factor VIII, and 15–20 preparations could be isolated with the same columns. Sepharose CL-4B in the presence of 0.5 M $CaCl_2$ gave better dissociation and separation of factor VIII from vWF than 0.25 M $CaCl_2$. Of the three protein absorbance peaks, the first closely paralleled the vWF, whereas contaminants eluted in two small peaks between 0.60 and 0.90 V_e/V_t. The factor VIII activity was consistently present as a broad peak (0.63–0.86 V_e/V_t) with a maximum between the two small absorbance peaks. Furthermore, factor VIII activity did not correlate with any protein band visible on the gels stained with Coomassie blue.

Table I: Purification of Human vWF from Factor VIII Concentrate

purification step	total protein (OD_{280})	total act. (antigen units)[a]	sp act. (units/OD_{280})	recovery (%)	purification (*n*-fold)
concentrate	17 300	34 900	2	(100)	(1)
Sepharose CL-4B	347	25 400	74	73	37
gelatin/agmatine–Sepharose	201	23 100	115	66	58
high calcium CL-4B	122	18 200	149	52	74

[a] vWF antigen was measured by ELISA.

vWF was stable as a $(NH_4)_2SO_4$ paste at −70 °C and readily dissolved in neutral saline buffers at protein concentrations of 5–10 mg/mL. The protein contained 190 units/mg vWF as measured by enzyme-linked immunoassay, 200 units/mg by immunoradiometric assay, and 220 units/mg by rocket electroimmunoassay. The ristocetin cofactor activity was 110 units/mg, and the residual factor VIII activity was 1.8 units/mg. A total of 750 mL of concentrate gave over 100 mg of vWF, corresponding to an overall recovery of more than 50% and a 74-fold purification from the original concentrate. This is equivalent to about a 10 000-fold purification from plasma.

Electrophoretic Analysis. Electrophoresis of the starting concentrate on composite gels showed a number of faint, slow-moving bands due to vWF (Figure 1, lane 2). The major proteins in this sample included a strong band due to fibronectin that migrated at the position of IgM, a doublet due to fibrinogen, and a less dense band migrating at the position of IgG. After the first Sepharose CL-4B column (lane 3), the vWF bands were much more prominent, but a significant contamination with fibronection and fibrinogen remained. The final preparation (lane 5) showed 11 vWF bands, designated multimers 1–11. These were the only bands that bound monospecific anti-vWF antibody on Western blots. Multimer 1 routinely migrated just behind IgM and fibronectin. Trace contaminants consisted of a small amount of fibrinogen (0.4–2%) and a trace amount of an unidentified protein that

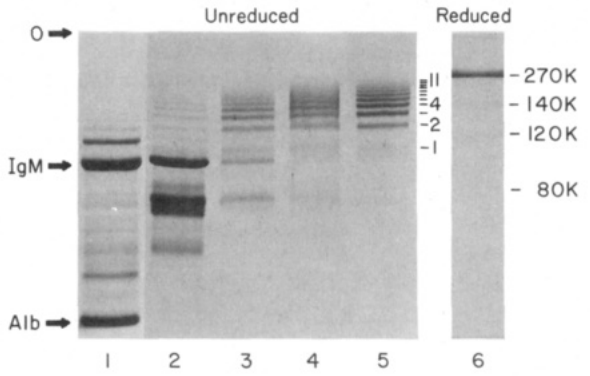

FIGURE 1: Electrophoretic analysis at different stages of vWF purification. Lanes 1–5 correspond to Coomassie blue stained composite gels ($NaDodSO_4$, urea, 1.6% polyacrylamide, 0.75% agarose) without reduction. Lane 6 shows Coomassie blue stained Laemmli 7.5% polyacrylamide gel of purified vWF under reducing conditions. Each sample well was 1.9 × 0.13 cm in cross-section. Samples were treated and gels run as described under Experimental Procedures. (Lane 1) Plasma from a patient with IgM myeloma (~100 μg). (Lane 2) Starting factor VIII concentrate (100 μg). (Lane 3) vWF pool from the first Sepharose CL-4B column (15 μg). (Lane 4) vWF pool from tandem gelatin– and agmatine–Sepharose columns (15 μg). (Lane 5) Purified vWF (14 μg). (Lane 6) Purified vWF after reduction (14 μg). Markers on the left show the position of the origin, IgM, and albumin. Markers in the right center indicate the vWF multimers numbers. Markers on the right show molecular weights.

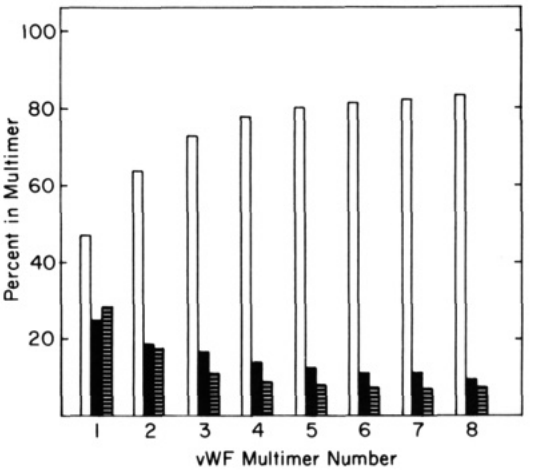

FIGURE 2: Composition of isolated vWF multimers. ^{125}I-vWF added to purified vWF was submitted to composite gel electrophoresis ($NaDodSO_4$, urea, 1.6% polyacrylamide, 0.7% agarose) to separate the multimers. The gel was sliced in 1.2-mm pieces, and each was counted. Slices containing the maximum of each ^{125}I-labeled multimer (~70 000 cpm) were boiled in the presence of 1% $NaDodSO_4$ and 5% β-mercaptoethanol and reelectrophoresed on a Laemmli 7.5% polyacrylamide gel. After being stained, dried, and autoradiographed, the bands at 270K, 140K, and 120K were cut out and counted. Results are expressed as the percent that each band contributed to the total radioactivity for that multimer. (Open) 270K subunit; (solid) 140K component; (barred) 120K component.

migrated at the position of IgM. Composite gels quantified by either Coomassie blue staining or radioactivity showed that vWF accounted for more than 95% of the total protein.

Following reduction and Laemmli gel electrophoresis, the final vWF preparation (Figure 1, lane 6) showed a major band at about 270K and two trace bands at 140K and 120K. There was also a very faint band at about 80K, three extremely faint bands at 40K–50K (probably fibrinogen), and a small amount of polypeptides that migrated at the front. The 270K, 140K, and 120K bands bound to monospecific anti-vWF antibody in similar proportion to their Coomassie blue staining, and no other band bound this antibody. Furthermore, two different monoclonal antibodies to human vWF were found to react in Western blots with the 140K and 270K bands but not with the 120K band. Scans of these bands stained with Coomassie blue showed that they added up to 96% of the total protein and accounted for 86.0, 9.9, and 4.0% of vWF, respectively. The 120K band consistently contained more ^{125}I than expected from Coomassie blue staining.

Distribution of Multimers in Purified vWF. Individual vWF multimers were resolved on composite gels and quantitated by four techniques, including scanning of the bands stained with Coomassie blue and three variations of ^{125}I-vWF detection. The results were similar for each technique. Scans of Coomassie blue and counts of ^{125}I-vWF in cut-out blue-stained bands, however, allowed quantification of higher multimers. Band number 1 represented 4% of the vWF, while bands 2–9 contained 13, 22, 22, 15, 10, 7, 4, and 2% of the protein, respectively. Bands 10 and 11 contained less than 1% of the total vWF. Average molecular weights were calculated from 10 different determinations. The molecular weight of band 1 was set at M, and each multimer was assumed to be an integral multiple of M. Thus, the number-average was $3.35M$, the weight-average was $4.30M$, and the Z-average was $5.26M$. Composite electrophoresis gels of fractions from the first preparative Sepharose CL-4B column showed that the 25% vWF loss at this step was primarily from multimer bands 1–3.

Composition of Multimers after Reduction. Electrophoretically purified vWF multimers contained only the 270K, 140K, and 120K bands, and trace contaminants were no longer present. vWF multimer number 1 was composed of 47% of the 270K subunit, 25% of the 140K subunit, and 28% of the 120K subunit, as estimated by radioactivity (Figure 2). The mole ratios in these bands were 1.00, 0.95, and 1.24, respectively. The composition of each of the larger multimers varied continuously as their band number increased. The 270K subunit comprised 64% of band 2, 72% of band 3, and reached a maximum of 83% in the highest multimers. Also, the 140K and 120K bands decreased in parallel to a level of 9 and 7%, respectively, in the highest multimers. Control experiments showed that vWF multimers isolated from composite gels were stable for at least several hours at 22 °C and that they migrated in the same position when reelectrophoresed on a composite gel. There was no evidence of contamination by other neighboring multimer bands, nor was there any transformation into either higher or lower mobility multimers.

Composition and Sequence Analysis. Table II shows the amino acid and carbohydrate compositions of the subunit of human von Willebrand factor. These calculations assume a subunit M_r of 270K as estimated by SDS gel electrophoresis. Recovery of amino acids was 86% by weight after corrections were made for moisture and ash in the sample.

The amino acid composition indicates that the protein contains approximately 39 methionyl residues per 270K subunit or 78 residues if the protein were composed of two nonidentical subunits of 270K. Therefore, cleavage at methionyl bonds with cyanogen bromide should give rise to about 40 fragments if the protein were composed of identical subunits. Five milligrams of the S-CM protein (ca. 20 nmol) was cleaved with cyanogen bromide, and the digest was separated into 12 fractions by HPLC size exclusion (Figure 3). Subsequent separation of individual fractions was achieved by reverse-phase HPLC on either a C3 or a C18 column. This is illustrated in Figure 4, which shows the separation of fraction 10 into several additional peaks, two of which were labeled peptide N and peptide C. All together, 41 major fragments were isolated, and three were regarded to be minor due to their poor yields and poor reproducibility of isolation. These data indicate

Table II: Amino Acid and Carbohydrate Compositions of Human von Willebrand Factor[a]

amino acids	residues/270K-dalton subunit	peptide N	peptide C
Asx	189		
Thr	114[b]		
Ser	131[b]	1.86	2.00
Glx	257		1.36
Pro	133	1.88	1.10
Gly	138		
Ala	106		
$^1/_2$-Cys[c]	146	0.82	3.10
Val	154[d]		
Met	39		
Ile	71[d]		
Leu	153	1.00	
Tyr	56		
Phe	61		
His	54		
Lys	88		3.00
Arg	96	1.19	1.15
Trp	43[e]		
homo-Ser		0.33	
total	2029	8	11

	content (%)
protein	81.3
carbohydrate	18.7
hexose	6.5
N-acetylhexosamine	8.7
N-acetylneuraminic acid	3.5

[a] By amino acid analysis of 24-, 48-, and 96-h acid hydrolysates of the whole S-CM protein. [b] Extrapolated to zero-time acid hydrolysis. [c] Analyzed as *S*-CM-Cys. [d] Taken from values of 96-h acid hydrolysis. [e] Analyzed by base hydrolysis according to Hugli and Moore (1972).

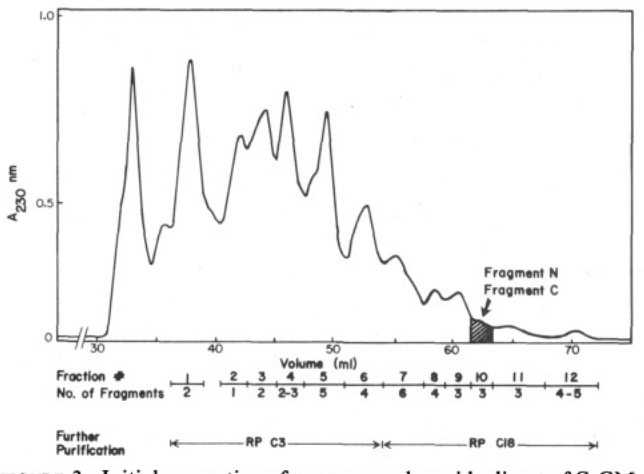

FIGURE 3: Initial separation of a cyanogen bromide digest of S-CM human von Willebrand factor by size-exclusion HPLC. Five milligrams of the digest was separated on three TSK columns connected in a series and equilibrated in 6 M guanidine hydrochloride and 10 mM sodium phosphate, pH 6.0. The elution was achieved at a flow rate of 0.5 mL/min, monitored at 230 nm. Fractions were made as shown and further separated by reverse-phase HPLC (not shown) on C3 and C18 columns.

that the 270K subunit is composed of one identical polypeptide chain. The composition of peptides N and C is also shown in Table II. Peptide C was the only peptide found to lack homoserine, indicating that it originates from the carboxyl-terminal end of the polypeptide chain.

The intact S-CM protein and peptides N and C were then subjected to sequence analysis. The background was fairly high in the sequence analysis of 10 nmol of S-CM protein due to its very high molecular weight. The major sequence, however, was unambiguously identified as Ser-Leu-Ser-Cys-Arg-Pro-Pro-Met-Val-Lys. This sequence was in good

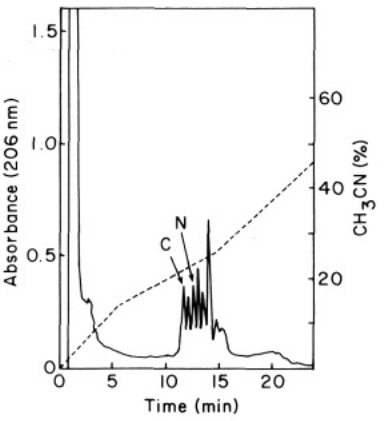

FIGURE 4: Fraction 10 was further purified by reverse-phase HPLC on a Synchropak RP-P column as described under Experimental Procedures and subjected to amino acid and sequence analysis.

Table III: Variation in Platelet Binding of Individual Multimers

	600 ng/mL total vWF		
multimer no.	bound (ng/mL)[a]	free (ng/mL)	ratio of bound/free
1	17	20	0.9
2	37	48	0.8
3	74	67	1.1
4	82	42	1.9
5	60	22	2.7
6	41	9	4.7
7	25	4	6.1
8	14	2	6.1
9–11	28	7	4.0
total	378	221	1.7
fraction	0.63	0.37	

[a] Calculations were performed as follows: amount of multimer *i* bound = (600 ng/mL)(fraction bound)(fraction of multimer *i* bound).

agreement with that reported by Hessel et al. (1984) and with the first five residues determined from 9 nmol of peptide N, which were found to be Ser-Leu-Ser-Cys-Arg. The remainder of peptide N was washed out of the sequencer cup after the first five cycles of Edman degradation.

An analysis on 12 nmol of peptide C gave a sequence of Glu-Cys-Lys-Cys-Ser-Pro-Arg-Lys-Cys-Ser-Lys. This sequence is in excellent agreement with the amino acid composition for this peptide (Table II) and provides additional evidence for this peptide originating from the carboxyl-terminal end of the protein. The sequence data provide strong evidence for the conclusion that the vWF subunit of 270K is composed of a single glycopeptide chain with an amino-terminal serine and a carboxyl-terminal lysine residue.

Relative Affinity of vWF Multimers for Platelets. The relative affinity of different vWF multimers for platelet binding sites was estimated by two independent techniques. In the first, purified vWF was allowed to react with platelets in the presence of ristocetin under the conditions of the platelet binding assay. Individual multimers from both platelet bound and unbound fractions were quantified. Table III shows results from a typical experiment performed with a low vWF concentration of 600 ng/mL and 10^8 platelets/mL. Under these experimental conditions, the platelet binding sites were present in large excess, and the ratio of bound to free multimers was a reflection of the apparent binding constant. When compared to the starting multimer distribution, the platelet-bound fraction was enriched in multimers ≥6 and depleted of multimers 1–3. Conversely, the unbound fraction was enriched in multimers 1–3 and depleted of multimers ≥6. Multimers 4 and 5 appeared in a transition zone. The amount of bound

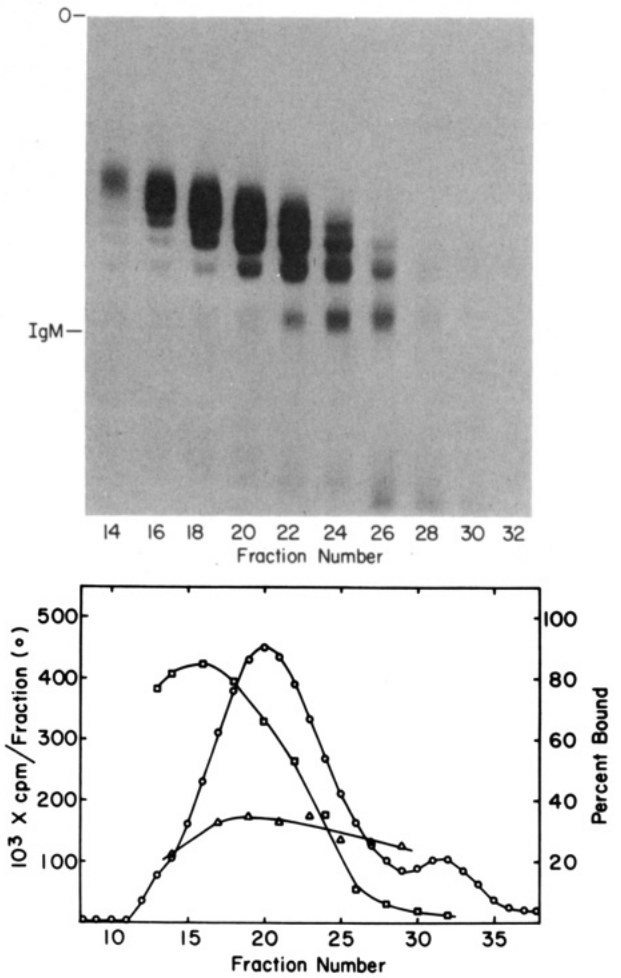

FIGURE 5: Analysis of the ristocetin-induced platelet binding of vWF gel filtered through Sepharose CL-2B. A sample of 200 μL of ^{125}I-vWF (15 μg) was applied to a Sepharose CL-2B column (0.7 × 50 cm), equilibrated in 0.15 M NaCl, 0.1% BSA, and 0.025 M Tris-HCl, pH 7.4. Fractions of 0.5 mL were collected, counted for radioactivity, tested with the binding assay, and analyzed by composite gel electrophoresis ($NaDodSO_4$, urea, 1.6% polyacrylamide, 0.75% agarose). Aliquots were also partially reduced by 10 mM β-mercaptoethanol, alkylated with *N*-ethylmaleimide, dialyzed, and assayed for their binding to the platelets. (Top panel) Autoradiograph of the gel showing multimeric distribution in fractions eluted from Sepharose CL-2B column. Each well contained a sample in 6 M urea, 1% $NaDodSO_4$, and 0.1 M Tris-HCl, pH 7, with purified vWF (~10 μg) as a carrier. (Bottom panel) (○) Elution profile of ^{125}I-vWF from Sepharose CL-2B; (□) binding of ^{125}I-vWF fractions; (△) binding of partially reduced and alkylated ^{125}I-vWF fractions. B/T was 63% with ^{125}I-vWF and 22% with partially reduced vWF.

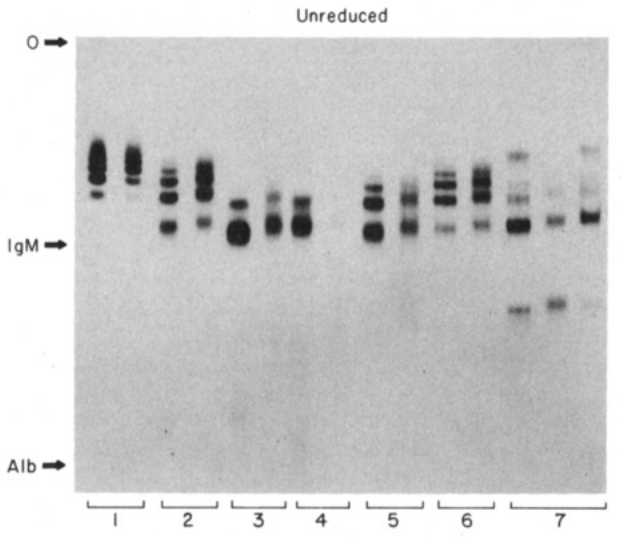

FIGURE 6: Autoradiograph after composite gel electrophoresis ($NaDodSO_4$, urea, 1.6% polyacrylamide, 0.75% agarose) of vWF digests and the portion that bound to platelets in the presence of ristocetin. Digests were performed at pH 8 with ^{125}I-vWF at the concentration of 50 μg/mL with an enzyme/substrate ratio of 1/50 for bovine thrombin, trypsin, subtilisin, thermolysin, and α-chymotrypsin. *S. aureus* V-8 protease was used at a 1/25 enzyme/substrate ratio. Digestion was stopped by diisopropyl fluorophosphate (5 mM final). Thermolysin digestion was also stopped by EDTA (10 mM final). Binding to platelets and extraction of bound material were performed as described under Experimental Procedures. Two lanes are shown for each enzyme (three for *S. aureus* V-8 protease). In each case, the left lane shows the digestion products, and the right shows the bound material that was extracted from platelets. For *S. aureus* V-8 protease, the third lane is the free material remaining in the supernatant. (1) The 20-h bovine thrombin digest (~14 000 cpm in both lanes); (2) 2-h tryptic digest (~12 000 cpm in both lanes); (3) 20-h tryptic digest (~15 000 cpm in both lanes); (4) 2-h subtilisin digest (16 000 cpm; 4000 cpm); (5) 20-h thermolysin digest (16 000 cpm, 10 000 cpm); (6) 20-h α-chymotrypsin digest (16 000 cpm; 10 000 cpm); (7) 20-h V-8 protease digest (16 000 cpm; 9000 cpm; 11 000 cpm). Markers show the position of the origin, IgM, and albumin.

and free vWF multimers and their ratios are also shown in Table III. Thus, the apparent binding constants of multimers 1–3 were similar, and these rose almost 8-fold higher for multimers ≥6.

In the second procedure, the vWF multimers were separated initially by gel filtration of Sepharose CL-2B and then assayed for binding to platelets in the presence of ristocetin (Figure 5). The highest specific binding to platelets (B/T = 78–85%) was associated with the fractions containing the highest molecular weight forms (fractions 13–18). This binding was relatively constant, even though there were significant changes in the multimer distribution. The striking decrease in platelet binding between fractions 18 and 26 was associated with a continuous decrease in vWF bands greater than 4 and an increase in bands 1–3. In contrast, partially reduced vWF obtained from these Sepharose CL-2B fractions gave relative constant specific binding and did not depend upon the vWF multimers present in the starting fractions (e.g., 34% in fraction 17 and 29% in fraction 25). The ratio bound/free was about 4 for the largest multimers and 0.5 for the smallest or partially reduced multimers. As found in the previous experiment, this was an 8-fold increase between smallest and largest multimers. Control experiments using ^{125}I-labeled native vWF and partially reduced vWF showed that platelet binding expressed as percent was independent of the final ligand concentration between 0.01 and 0.5 μg/mL. It was 63% with ^{125}I-vWF as starting material and 22% with the partially reduced and alkylated form. In addition, all the partially reduced fractions and partially reduced vWF had identical mobility by composite gel electrophoresis.

Proteolysis of Purified vWF and Binding to Platelets. Among the 11 proteases tested, *S. aureus* V-8 protease resulted in a rather specific, limited proteolysis of native vWF. This enzyme generated two major fragments: one was a dimer of polypeptides of about 160K and readily bound to platelets in the presence of ristocetin; the other was a dimer of polypeptides of about 100K with anomalous electrophoretic mobility but without ristocetin-induced platelet binding. These two fragments are shown in Figure 6, lane 7, as the fastest and slowest moving bands, respectively. The intermediate bands represent partially digested vWF and also retain their platelet binding behavior.

Mild proteolysis by trypsin, subtilisin, thermolysin, or α-chymotrypsin produced stepwise integral changes in vWF mobility (Figure 6, lanes 2–6). These digests retained mul-

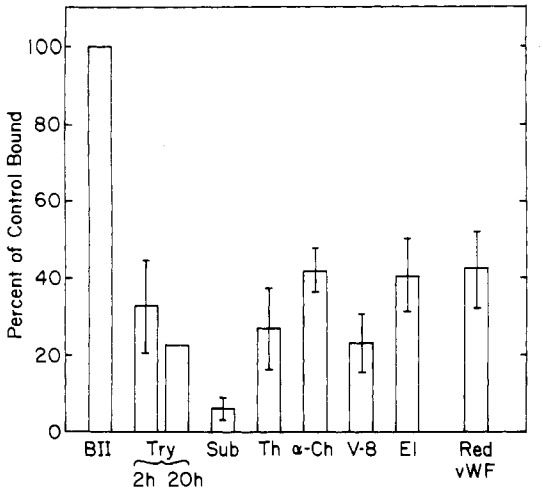

FIGURE 7: Ristocetin-induced platelet binding of enzymatic digests of ^{125}I-vWF and partially reduced vWF. Digests were performed with ^{125}I-vWF at 50 μg/mL, as described in Figure 6. Partially reduced vWF was prepared as described under Experimental Procedures and used after labeling. Binding to platelets in the presence of ristocetin (1.2 mg/mL) was performed at a final concentration of 0.1 μg/mL labeled protein and 10^8 cells/mL. Results are expressed as the percent of control (undigested ^{125}I-vWF) binding and are the average ± SD of several determinations (n) each done in duplicate. (BII) The 20-h bovine thrombin digest (n = 2); (Try 2h) 2-h tryptic digest (n = 4); (Try 20h) 20-h tryptic digest (single determination); (Sub) 2-h subtilisin digest (n = 4); (Th) 20-h thermolysin digest (n = 4); (α-Ch) 20-h α-chymotryptic digest; (V-8) 20-h V-8 protease digest (n = 4); (El) 20-h elastase digest (n = 3); (Red vWF) partially reduced vWF (n = 6).

timer-like patterns analogous to the first three or four multimers of native vWF. After reduction, Laemmli gels from each of these digests demonstrated no intact 270K subunit as the enzymes had already produced a complex but characteristic pattern of multiple small fragments. Each digest was tested for ristocetin-induced platelet binding, and the platelet-bound fraction was examined by gel electrophoresis. Under the conditions described in Figure 7, trypsin, thermolysin, α-chymotrypsin, and elastase decreased binding to 27–42% of control, and extracts of the bound fractions were electrophoretically almost identical with the whole digest (Figure 6, lanes 2, 3, 5, and 6). By comparison, partially reduced ^{125}I-vWF demonstrated 42% of native vWF binding.

In the case of *S. aureus* V-8 protease digests, platelet binding was only 23% of control, but as stated above, there was a large fragment cleaved from vWF that did not bind to platelets. Digests by subtilisin showed very little platelet binding (6% of control) even though the electrophoretic appearance before reduction was similar to that of digests with other proteases. Bovine factors IXa and Xa, human factor XIIa, bovine and human thrombin, and mast cell protease did not appear to modify native vWF as both platelet binding and gel electrophoretic patterns were unchanged.

Discussion

The present data provide strong evidence for the conclusion that vWF is a multimer composed of subunits of M_r 270K, in addition to some fragments of the M_r 270K subunits. This conclusion was supported by the cyanogen bromide fragmentation experiments and the partial amino acid sequence analysis. It is also consistent with the amino acid sequence predicted from the cDNA (Sadler et al., 1985). Partial amino acid sequence analysis and experiments with the cDNA indicate the presence of about 2050 amino acid residues per 270K subunits. This is in good agreement with the data presented in Table II, except for the tryptophan content, which is about 2-fold greater than that estimated from amino acid sequence or cDNA data (Sadler et al., 1985; Titani et al., 1986). CM-Cys accounts for 7.2% of the amino acid residues, which is significantly higher than the 3.1% expected from a random distribution of nucleotide triplets with observed mammalian base composition (King & Jukes, 1969). The total amino acid composition, however, is similar to that reported by others (Legaz et al., 1973; Shapiro et al., 1973).

The percentage of *N*-acetylhexosamine in vWF is higher than usually reported, but it is known that over or under hydrolysis may give artifactually low values (Gardell, 1957). The molecular weight of this subunit calculated from the amino acid data presented in Table II is 222K. This brings the total molecular weight of the subunit to about 270K, assuming 18.7% carbohydrate.

These experiments were made possible by the development of a rapid, highly reproducible, large-scale preparation of human vWF starting from commercial factor VIII concentrates. The important difference between this and other methods is the intermediate adsorption of contaminants with gelatin–Sepharose and agmatine–Sepharose. These steps made it possible to obtain high yields of the smaller multimers of vWF. Each preparation produced over 100 mg of vWF, which was greater than 95% pure.

Most investigators (Counts et al., 1978; Hoyer, 1981; Meyer et al., 1980; Fass et al., 1978) have found vWF multimers to be a regular array of bands very similar to the pattern that we observed. In addition, completely reduced human vWF has been described as homogeneous on $NaDodSO_4$ gel electrophoresis. This has led several to suggest that the complex is assembled from identical or nearly identical subunits. Ruggeri and Zimmerman (1981) observed electrophoretic microheterogeneity of plasma vWF. They found that each multimer could be resolved into a triplet on discontinuous gel electrophoresis, and they concluded that vWF assembly must be more complex than a simple linear polymerization of a single protomer. Ruggeri et al. (1982) have extended these observations to describe variants of von Willebrand's disease that are characterized by different patterns of electrophoretic microheterogeneity. Heterogeneity of purified vWF has also been described by electrophoresis both before and after reduction. Ohmori et al. (1982) have observed an irregular Coomassie blue staining pattern after electrophoresis of human vWF purified from factor VIII concentrate. These authors also postulated that vWF was assembled from nonidentical subunits. Gorman and Ekert (1978) found that reduced vWF contained the 270K subunit and six additional smaller components and suggested that vWF may be composed of distinct polypeptide chains. Martin et al. (1981) also found minor components of 197K, 174K, and 154K present in constant proportion in vWF multimers that had been partially resolved on Sepharose CL-2B; they postulated that these did not represent in vitro proteolysis.

After complete reduction and $NaDodSO_4$ gel electrophoresis, we have observed that purified vWF contained a major band of 270K and minor bands of 140K and 120K. These two smaller polypeptides reacted with monospecific antibodies to human vWF and were associated with vWF multimers in the presence of 6 M urea and 1% $NaDodSO_4$. Reducing agents were required to separate them from the 270K subunit, and therefore, the 140K and 120K components must be covalently assembled into the vWF complex, presumably by disulfide bonds. In addition, the observation that two monoclonal antibodies specifically react with the 140K component and 270K subunit establishes that the 140K component must be derived

from the 270K subunit. Similar components were consistently observed during large-scale preparations of bovine vWF from fresh plasma collected in the presence of several protease inhibitors. These were also shown to be covalently linked into the vWF complex and had very similar molecular weights. The 140K and 120K components were quantified in human preparations, and we found a surprising nonuniform distribution between different multimers. It seems likely that the 140K and 120K components arise from limited proteolysis of the 270K subunit and that this occurred prior to the purification of vWF. If it is assumed, as previously suggested by other investigators (Hamilton et al., 1985), that each individual vWF multimer consists of a linear arrangement of 270K subunits, then the 140K and 120K proteolytic derivatives may be located at the ends of the multimers. Since we found that the smallest multimer (i.e., number 1) contains equimolar amounts of 270K, 140K, and 120K, the minimum molecular weight of this multimer is ~500K. However, these results require that multimer 1 is not identical with the difference between two adjacent multimers (the basic assembly protomer composed of intact subunits). In contrast to previous estimates, this is based on quantification and molecular weight determination of completely reduced vWF protein.

The structure–function relationships of vWF were examined by employing limited proteolysis of the protein and analyzing the resultant products by electrophoresis and platelet binding. Early digests by several different enzymes produced fragments that retained a "multimer-like" pattern and still bound to platelets. Each of these "multimers" was composed of numerous disulfide-linked polypeptides. The presence of intact 270K subunit was not required for the multimer-like pattern nor for platelet binding. Martin et al. (1981) have isolated a 116K tryptic fragment that contained 5% of the starting ristocetin cofactor activity, and after reduction, this fragment was composed of 76K, 46K, and 43K polypeptides. This fragment was not identified in the present studies. This may be due to less extensive digestions in the present investigation and/or to the use of affinity-purified trypsin that was free of α-chymotrypsin (Fujikawa & McMullen, 1983). *S. aureus* V-8 protease produced very limited proteolysis and separated vWF into two domains with strikingly different properties, but each composed of one primary polypeptide. Subtilisin hydrolyzed vWF near the V-8 protease site and produced a fragment with the same anomalous mobility. Subtilisin also hydrolyzed the other domain of vWF into a number of small fragments, which retained little platelet binding activity. The anomalous mobility of a large vWF domain, produced by either V-8 protease or subtilisin, appeared to be a highly characteristic property.

Experiments in which different multimers were allowed to react with an excess of platelet binding sites have shown that the larger multimers have at least an 8-fold higher binding affinity. This is a minimal estimate because extraction of the platelet-bound vWF may underrepresent the more strongly bound multimers. Partially reduced vWF bound to platelets at 42% of the levels observed for the native protein. Similar results were found with partially reduced vWF prepared from either large or small multimers. Platelet binding by products of mild proteolysis with trypsin, thermolysin, α-chymotrypsin, or elastase was also about 40% of control levels. In each of these experiments, platelet binding sites were in large excess, and electrophoresis verified that the bound protein was representative of the total labeled protein. Therefore, the percent binding may be used as a measure of the apparent binding constant. These results show that two independent modifications of native vWF (either partial reduction or proteolysis) each decrease the apparent binding constant to very similar levels. These data indicate that the platelet binding site is essentially intact after either modification and that the primary change is a decrease in molecular size. Since we have found that vWF is constructed from an identical repeating subunit, we also suggest that vWF is a multivalent molecule with repeating platelet binding domains and that these can react at more than one platelet site. Multimers larger than number 4 or 5 may be required to span the distance between two adjacent platelet sites. Our data do not allow us to establish whether these two platelet sites are on the same or adjacent platelets. According to this hypothesis, partial reduction and proteolysis would produce essentially monovalent molecules that are too small to interact with more than one platelet site. Thus, vWF may be similar to other binding proteins such as IgG and IgM, where multivalency has been shown to result in large increases in the apparent binding constant (Hornick & Karush, 1972).

ACKNOWLEDGMENTS

We are indebted to Dr. W. Kisiel for helpful discussions and to Dr. D. Meyer for critical revision of the manuscript. We are grateful to Lee Hendrickson and Santosh Kumar for excellent technical assistance, to Roger D. Wade for amino acid analysis, to Lowell H. Ericsson for sequence analysis, and to Lois Swenson for preparing illustrations. We thank Valerie Charles and Lois Swenson for typing the manuscript.

Registry No. vWF, 9001-27-8.

REFERENCES

Allain, J. P., Cooper, H. A., Wagner, R. H., & Brinkhous, K. M. (1975) *J. Lab. Clin. Med. 85*, 318–328.

Ardaillou, N., Girma, J.-P., Meyer, D., Lavergne, J. M., & Larrieu, M. J. (1978) *Thromb. Res. 12*, 817–830.

Atichartakarn, V., Marder, V. J., Kirby, E. P., & Budzynski, A. Z. (1978) *Blood 51*, 281–297.

Brauer, A. W., Margolies, M. N., & Haber, E. (1975) *Biochemistry 14*, 3029–3035.

Bridgen, P. J., Cross, G. A. M., & Bridgen, J. (1976) *Nature (London) 253*, 613–614.

Brinkhous, K. M., Graham, J. E., Cooper, H. A., Allain, J. P., & Wagner, R. H. (1975) *Thromb. Res. 6*, 267–272.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Freeman, San Francisco.

Casillas, G., Simonetti, C., Lazzari, M., & Kempfer, A. (1980) *Thromb. Haemostasis 43*, 68.

Chan, V., & Chan, T. K. (1982) *Thromb. Haemostasis 48*, 177–181.

Counts, R. B., Paskell, S. L., & Elgee, S. K. (1978) *J. Clin. Invest. 62*, 702–709.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

DiMinno, G., Shapiro, S. S., Catalano, P. M., De Marco, L., & Murphy, S. (1983) *Blood 62*, 186–190.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.

Engvall, E. (1980) *Methods Enzymol. 70*, 419–439.

Ericsson, L. H., Wade, R. D., Gagnon, J., MacDonald, R. M., Granberg, R. R., & Walsh, K. A. (1977) in *Solid Phase Method in Protein Sequence Analysis* (Previero, A., & Coletti-Previero, M. A., Eds.) p 137, Elsevier/North-Holland, New York.

Fass, D. N., Knutson, G. J., & Bowie, E. J. W. (1978) *J. Lab. Clin. Med. 91*, 307–320.

Fraker, P. J., & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun. 80*, 849–857.
Fujikawa, K., & McMullen, B. (1983) *J. Biol. Chem. 258*, 10927–10933.
Fujikawa, K., Walsh, K. A., & Davie, E. W. (1977) *Biochemistry 16*, 2270–2278.
Fujimoto, T., Ohara, S., & Hawiger, J. (1982) *J. Clin. Invest. 69*, 1212–1222.
Gardell, S. (1957) *Methods Biochem. Anal. 6*, 289–317.
Gorman, J. J., & Ekert, H. (1978) *Thromb. Res. 12*, 341–352.
Gralnick, H. R., Williams, S. B., & Morisato, D. K. (1981) *Blood 58*, 387–397.
Gralnick, H. R., Williams, S. B., & Rick, M. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2771–2774.
Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.
Hamilton, K. K., Fretto, L. J., Grierson, D. S., & McKee, P. A. (1985) *J. Clin. Invest. 76*, 261–270.
Harrison, R. L., & McKee, P. A. (1983) *Blood 62*, 346–353.
Hessel, B., Jornvall, H., Thorell, L., Soderman, S., Larsson, U., Egberg, N., Blomback, B., & Holmgren, A. (1984) *Thromb. Res. 35*, 637–651.
Hjort, P., Rapaport, S. I., & Owren, P. A. (1955) *J. Lab. Clin. Med. 46*, 89–97.
Hornick, C. L., & Karush, F. (1972) *Immunochemistry 9*, 325–405.
Hoyer, L. W. (1981) *Blood 58*, 1–13.
Hoyer, L. W., & Shainoff, J. R. (1980) *Blood 55*, 1056–1059.
Hugli, T. E., & Moore, S. (1972) *J. Biol. Chem. 247*, 2828–2834.
Kao, K. J., Pizzo, S. V., & McKee, P. A. (1979) *J. Clin. Invest. 63*, 656–664.
King, J. L., & Jukes, T. H. (1969) *Science (Washington, D.C.) 164*, 788–798.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laurell, C. (1966) *Anal. Biochem. 15*, 45–52.
Legaz, M. E., Schmer, G., Counts, R. B., & Davie, E. W. (1973) *J. Biol. Chem. 248*, 3946–3955.
Mahoney, W. C., & Hermodson, M. A. (1980) *J. Biol. Chem. 255*, 11199–11203.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Martin, S. E., Marder, V. J., Francis, C. W., Loftus, L. S., & Barlow, G. H. (1980) *Blood 55*, 848–858.
Martin, S. E., Marder, V. J., Francis, C. W., & Barlow, G. H. (1981) *Blood 57*, 313–323.
Meyer, D., & Baumgartner, H. R. (1983) *Br. J. Haematol. 54*, 1–9.
Meyer, D., Obert, B., Pietu, G., Lavergne, J. M., & Zimmerman, T. S. (1980) *J. Lab. Clin. Med. 95*, 590–602.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Ohmori, K., Fretto, L. J., Harrison, R. L., Switzer, M. E., Erickson, H. P., & McKee, P. A. (1982) *J. Cell Biol. 95*, 632–640.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Perret, B. A., Furlan, M., & Beck, E. A. (1979) *Biochim. Biophys. Acta 578*, 164–174.
Pietu, G., Cherel, G., Marguerie, G., & Meyer, D. (1984) *Nature (London) 308*, 648–649.
Proctor, R. R., & Rapaport, S. I. (1961) *Am. J. Clin. Pathol. 36*, 212–219.
Ruggeri, Z. M., & Zimmerman, T. S. (1980) *J. Clin. Invest. 65*, 1318–1325.
Ruggeri, Z. M., & Zimmerman, T. S. (1981) *Blood 57*, 1140–1143.
Ruggeri, Z. M., Bader, R., & De Marco, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6038–6041.
Shapiro, G. A., Anderson, J. C., Pizzo, S. V., & McKee, P. A. (1973) *J. Clin. Invest. 52*, 2198–2210.
Sixma, J. J., Sakariassen, K. S., Beeser-Vissel, N. H., Ottenhof-Rovers, M., & Bolhuis, P. A. (1984) *Blood 63*, 128–139.
Titani K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., & Fujikawa, K. (1986) *Biochemistry* (fourth paper of four in this issue).
Towbin, H., Stachelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Tschopp, T. B., Weiss, H. J., & Baumgartner, H. R. (1974) *J. Lab. Clin. Med. 83*, 296–300.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.