# Limited Proteolysis of Human von Willebrand Factor by *Staphylococcus aureus* V-8 Protease: Isolation and Partial Characterization of a Platelet-Binding Domain†

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ABSTRACT: Purified human von Willebrand factor (vWF) was digested with *Staphylococcus aureus* V-8 protease, and specific domains interacting with platelets were isolated and characterized. Amino acid sequence analysis and sodium dodecyl sulfate gel electrophoresis demonstrated that the digestion proceeded primarily by a single cleavage of the native 270K subunit between an internal Glu–Glu peptide bond. This produced an integral stepwise degradation of the multimers of vWF with a concomitant accumulation of bands with mobility similar to that of the smaller molecular weight vWF multimers. The immediate precursor of the final products contained equimolar amounts of 270K subunit and of two polypeptides (170K and 110K). The cleavage of the remaining 270K subunit converted vWF into two main fragments (fragments II and III). These fragments were isolated by ion exchange chromatography, characterized, and assayed for platelet binding in the presence of ristocetin. Fragment III is a dimer of 315K composed primarily of two chains of 170K. Amino acid sequence analysis indicated that it originated from the amino-terminal portion of the 270K subunit and contained 11% of the original ristocetin cofactor activity. Also, it binds to platelets at the same specific sites as native vWF and shows a platelet binding pattern similar to that of partially reduced vWF (500K). Fragment II is a dimer of 235K composed of two identical chains of 110K. Amino acid sequence analysis indicated that it originated from the carboxyl-terminal portion of the 270K subunit and lacked ristocetin cofactor activity. Also, it does not bind to platelets or inhibit the binding of $^{125}$I-vWF in the presence of ristocetin. These data led to a model for the vWF structure in which the 270K subunits are linked by disulfide bonds that alternate between the two carboxyl-terminal and two amino-terminal regions of the molecule in a head-to-head and tail-to-tail manner.

Human von Willebrand factor (vWF)[1] is a glycoprotein present in plasma that is involved in the adhesion of platelets to the subendothelium and platelet aggregation. It is a polyfunctional molecule acting as a bridge between binding sites (Meyer & Baumgartner, 1983) on the subendothelium (Sakariassen et al., 1979) and the platelet membrane. The presence of an inducer is required for the binding of vWF to the platelet membrane, which involves distinct platelet membrane glycoproteins. In the presence of ristocetin, vWF binds to glycoprotein Ib (Nurden & Caen, 1975; Moake et al., 1980), while in the presence of thrombin or ADP, vWF binds to glycoproteins IIb and IIIa (Fujimoto et al., 1982; Ruggeri et al., 1982).

Various reports have established that vWF consists of a series of multimers ranging in size from $1 \times 10^6$ to $12 \times 10^6$ daltons (Hoyer & Shainoff, 1980; Ruggeri & Zimmerman, 1980; Chopek et al., 1986). Complete reduction of purified preparations leads to the formation of a predominant subunit estimated to be 260K (Legaz et al., 1973; Olson et al., 1977; Counts et al., 1978; Chopek et al., 1986). We and others have reported minor amounts of lower molecular weight components in vWF, suggesting that it also contains smaller nonidentical subunits (Legaz et al., 1973; Gorman & Ekert, 1978; Martin et al., 1981) with a nonuniform distribution between different vWF multimers (Chopek et al., 1986). Studies on the structure of vWF revealed that a dimer formed by association of 260K subunits is the basic unit of the vWF molecule (Counts et al., 1978). The assembly protomer, i.e., the difference between two consecutive multimers, may be either this dimer (Counts et al., 1978; Perret et al., 1979; Furlan et al., 1979) or a tetramer (Meyer et al., 1980; Ruggeri & Zimmerman, 1980). The multimeric series can be partially disassembled by mild reduction (Counts et al., 1978), carbohydrate modifications (Gralnick et al., 1983), or limited proteolysis (Chopek et al., 1986).

Recently, amino acid sequence analysis (Titani et al., 1986; Chopek et al., 1986) and cDNA cloning (Sadler et al., 1985; Shelton-Inloes et al., 1986) have indicated that the subunit of von Willebrand factor is composed of a single chain containing 2050 amino acids and 22 carbohydrate chains that are O- and N-linked. This yields a subunit structure of about 270K for human von Willebrand factor.

To date, few studies have been undertaken to correlate the various vWF functions with specific domains. Functional inhibition of monoclonal antibodies has established that a portion of the molecule is involved in platelet adhesion to the

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[1] Abbreviations: vWF, von Willebrand factor; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; $V_e/V_t$, elution volume at the protein peak ($V_e$) divided by the total volume of packed column bed ($V_t$).

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subendothelium (Meyer et al., 1981, 1984). Using tryptic digests of vWF, Martin et al. (1980) identified a small fragment of 116K possessing ristocetin cofactor activity. Sixma et al. (1984) using a series of selected monoclonal antibodies to vWF as probes of functional domains and tryptic fragmentation of vWF confirmed the presence of the binding site to ristocetin-treated platelets on a 116K species. They also identified a collagen interacting domain on an untreated 48K polypeptide. We have recently found using a large series of proteases that vWF fragments devoid of the 270K subunit are sufficient for binding to the platelet membrane (Chopek et al., 1986). In this paper, a detailed study of vWF degradation by *Staphylococcus aureus* V-8 protease is presented. The purification and characterization of two main vWF fragments are described, and their platelet-binding ability is compared to that of vWF.

## Experimental Procedures

*Materials.* The following were purchased from the suppliers indicated: Sephadex, Sepharose, DEAE-Sephacel, and high and low molecular weight standards (Pharmacia Fine Chemicals, Piscataway, NJ); human fibrinogen (Calbiochem-Behring Corp., La Jolla, CA); thyroglobulin (Helix Biotech, Ltd., Richmond, B.C., Canada); agarose [Sea-Kem HGT (P)], Gelbond (Marine Colloid, Rockland, ME); Iodo-gen (Pierce Chemical Co., Rockford, IL); Kodak-X-Omat AR film (Eastman Kodak Co., Rochester, NY); DFP (Aldrich Chemical Co., Milwaukee, WI); BSA type A-5003 (Sigma Chemical Co., St. Louis, MO); ristocetin (H. Lundbeck Co., Copenhagen, Denmark); *S. aureus* V-8 protease (Miles Laboratories, Inc., Elkhart, IN). Human factor VIII concentrate was kindly donated by Dr. Henry Kingdon of Hyland Therapeutics, Division of Travenol Laboratories. Plasma from a patient with IgM myeloma was a generous gift from Dr. B. Gilliland, University of Washington, and rabbit myosin was from Dr. E. Fischer, University of Washington.

*Purification and Characterization of Human von Willebrand Factor.* Human vWF was purified from factor VIII concentrate in the presence of multiple protease inhibitors as previously described (Chopek et al., 1986). The final product was stored as a 40% $(NH_4)_2SO_4$ precipitate at −70 °C. Aliquots were dissolved and dialyzed against buffer overnight. vWF protein concentrations were estimated from absorbance at 280 nm minus 1.7 times absorbance at 320 nm (Cantor & Schimmel, 1980) with an extinction coefficient $E_{280} = 10$. vWF antigen was estimated by enzyme-linked immunoassay (Chopek et al., 1986) or immunoradiometric assay (Ardaillou et al., 1978). Rocket immunoelectrophoresis was performed in the laboratory of Dr. R. Edson, University of Minnesota, by a modification of the method of Laurell (1966). Ristocetin cofactor activity was measured in the laboratory of Dr. R. Counts, Puget Sound Blood Center, according to the method of Brinkhous et al. (1975). The vWF used in this study contained an average of 190 antigen units/mg and 110 units/mg ristocetin cofactor activity when compared to a normal pool plasma defined as 1 unit/mL of each entity.

*Partial Reduction and Alkylation of vWF.* vWF (1 mg/mL) in 0.3 M Tris-HCl, pH 8.5, was reduced by 10 mM β-mercaptoethanol at 22 °C for 30 min (Chopek et al., 1986). The reaction was stopped with 20 mM *N*-ethylmaleimide. After 30 min in the dark, the solution was gel-filtered through Sephadex G-25. The fractions containing protein were pooled and dialyzed overnight against 0.025 M Tris-HCl, pH 7.4. The final product was analyzed by electrophoresis in a continuous $NaDodSO_4$–3.3% polyacrylamide gel and in a discontinuous $NaDodSO_4$–gradient polyacrylamide gel (3.5–12.5%). It had a molecular weight of 450K–500K without additional reduction. The specific ristocetin cofactor activity of this partially reduced vWF was 1 unit/mg.

*Formaldehyde-Fixed Human Platelets.* Formaldehyde-fixed human platelets were prepared according to the method of Allain et al. (1975). The final suspension in 0.15 M NaCl, 0.1% Nonidet P-40, 0.02% $NaN_3$, 0.1% BSA, and 0.025 M Tris-HCl, pH 7.4, was adjusted at 750 000 cells/μL, and aliquots were kept frozen at −70 °C.

*Labeling.* Proteins were labeled by the Iodo-gen method with $Na^{125}I$ (Fraker & Speck, 1978).

*Slab Gel Electrophoresis.* $NaDodSO_4$–polyacrylamide gel electrophoresis was performed with the discontinuous system of Laemmli (1970) or the continuous system of Weber and Osborn (1969). Composite gel (0.1% $NaDodSO_4$, 6 M urea, 1.6% polyacrylamide, 0.75% agarose) electrophoresis was performed as previously described (Chopek et al., 1986). Autoradiographs of dried gels were obtained by exposing preflashed films at −20 °C with an intensifying screen.

*Quantitative Electrophoresis.* Quantitative estimation of the electrophoretic bands was performed as previously described (Chopek et al., 1986) by scanning autoradiographs and Coomassie blue stained gels or by cutting out Coomassie blue stained, dried bands and counting associated radioactivity. Control experiments showed that staining by Coomassie blue was proportional to protein concentration over the range used in these experiments.

*Immobilization of S. aureus V-8 Protease. S. aureus* V-8 protease (V-8 protease) was immobilized onto CNBr-activated Sepharose CL-2B according to the method of March et al. (1974). The enzyme (10 mg) was coupled to 10 mL of beads with greater than 90% efficiency as determined by supernatant absorbance. The beads were washed and stored in 0.15 M NaCl, 0.02% $NaN_3$, and 0.02 M imidazole hydrochloride, pH 6.8, at 4 °C.

*Digestion of vWF by Solid-Phase V-8 Protease.* In a typical experiment, 8 mL of V-8 protease–Sepharose CL-2B (7–8 mg of enzyme) was packed in a column (1.5 × 30 cm) and equilibrated in 0.05 M NaCl, 0.01 M EDTA, and 0.05 M Tris-HCl, pH 7.8. Purified vWF (40 mg) diluted in 30 mL of the same buffer was loaded onto the column at 22 °C and recirculated at about 60 mL/h for 20 h. The column was eluted with buffer, and fractions containing protein were pooled. In order to prevent the possibility of further enzymatic degradation, the pooled fractions were made 10 mM DFP by adding 1/10 volume of freshly diluted solution. After extensive washing, the immobilized enzyme was stored in 0.15 M NaCl, 0.02% $NaN_3$, and 0.02 M imidazole hydrochloride, pH 6.8, at 4 °C. It could be used several times without apparent loss of activity. For quantitative data, $^{125}I$-vWF at $4 \times 10^6$ cpm/mg was digested, and samples were removed at selected times (0–28 h), made 10 mM DFP, submitted to reduced and unreduced $NaDodSO_4$ gel electrophoresis, and quantitated.

*Purification of Digestion Products.* The products of digestion by V-8 protease–Sepharose CL-2B (40 mg) were dialyzed against 0.05 M ammonium acetate, pH 5.8, and then loaded onto a 20-mL column of DEAE-Sephacel equilibrated in the same buffer. After being washed at 25 mL/h (140 mL), the adsorbed proteins were eluted by 120 mL of a linear gradient of ammonium acetate, pH 5.8, from 0.05 to 0.5 M. Fractions were pooled, adjusted to neutral pH, and concentrated by 40% $(NH_4)_2SO_4$ precipitation or lyophilization. In a modified procedure, digests were dialyzed against 0.075 M NaCl and 0.1 M $NaH_2PO_4$, pH 5.8, and loaded on the DEAE-Sephacel column equilibrated in the same buffer. After

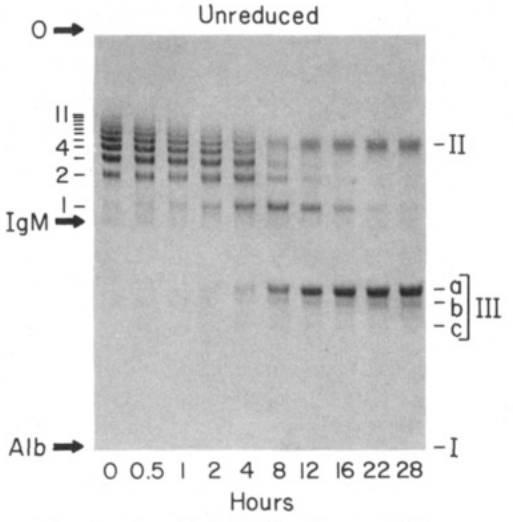


FIGURE 1: Digestion of purified vWF by *S. aureus* V-8 protease. The unreduced digestion products were run on composite gels ($NaDodSO_4$, urea, 1.6% polyacrylamide, 0.75% agarose) and stained with Coomassie blue. $^{125}$I-vWF (1.5 mg/mL, $6 \times 10^6$ cpm/mL) was digested at pH 7.8, 22 °C, with enzyme–Sepharose 2B and an enzyme/substrate ratio of 1:5. At selected times, samples (100 μL) were taken, treated with DFP, made 6 M urea and 1% $NaDodSO_4$, and subjected to electrophoresis on composite gels. Each well contained 5 μg of protein. The time is indicated at the bottom of the gel. Markers (left) show the position of the origin, IgM, and albumin. The arabic numbers (left) identify vWF multimers. Roman numerals (right) correspond to the digestion products. Fragment III is a triplet (IIIa, IIIb, IIIc).

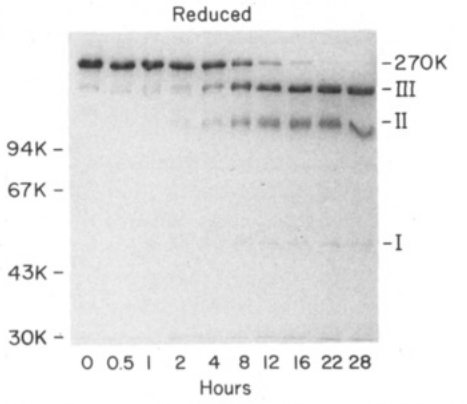


FIGURE 2: Digestion of purified vWF by *S. aureus* V-8 protease. The reduced digestion products were run on polyacrylamide gels (7.5%) and stained with Coomassie blue. Samples (50 μL) from Figure 1 were also made 0.125 M Tris-HCl, pH 6.8, and 5% β-mercaptoethanol and boiled for 2 min. Each well contained 5 μg of protein. The time is indicated at the bottom of the gel. Markers (left) show the position of molecular weight standards. The positions of the 270K subunit and the digestion products are indicated.

being washed, the unadsorbed proteins were precipitated by 40% $(NH_4)_2SO_4$, dissolved in a minimum volume of 0.15 M NaCl and 0.025 M Tris-HCl, pH 7.4, and separated by gel filtration on a Sephadex G-150 column (1.5 × 90 cm) equilibrated in the same buffer. The adsorbed fragments on DEAE-Sephacel were eluted with 0.3 M NaCl and 0.1 M $NaH_2PO_4$, pH 5.8, and dialyzed against 0.15 M NaCl and 0.025 M Tris-HCl, pH 7.4. Final products were stored at −70 °C.

*Amino Acid Sequence Analysis.* Automated amino acid sequence analysis was performed on a Beckman sequencer (Model 890C) following alkylation of the reduced protein with iodoacetic acid as described (Chopek et al., 1986).

*Platelet Binding Assays in the Presence of Ristocetin.* Platelet binding assays were performed at 22 °C in the presence of ristocetin as previously described (Chopek et al., 1986). The $^{125}$I-labeled protein (0.5–0.9 μCi/μg) was incubated at 0.1 μg/mL with fixed platelets ($10^8$ cells/mL) and ristocetin at 1.2 mg/mL. Separation of bound from free $^{125}$I-labeled protein, estimation of the specific binding, control experiments, and analysis of the platelet-bound material were performed as previously described (Chopek et al., 1986). Competitive inhibition of binding by unlabeled protein was performed with a modified assay. Platelets were incubated with a constant concentration of $^{125}$I-labeled protein (0.1 μg/mL final) that had been diluted into selected amounts of unlabeled material. The reaction was started by adding ristocetin (1.2 mg/mL final). Binding isotherms were determined by an assay system in which increasing concentrations of trace-labeled protein (0.005 μCi/μg) were used instead of constant amounts of highly labeled protein.

## RESULTS

*Proteolysis of vWF by S. aureus V-8 Protease. S. aureus* V-8 protease was coupled to Sepharose CL-2B so that it could be readily removed from the reaction mixture. Under these conditions, enzyme/substrate ratios of 1:4 or 1:6 gave reaction rates similar to those obtained in solution with ratios of 1:25 or 1:50. Composite gel electrophoresis (Figure 1) showed a gradual loss of the vWF multimers and formation of two main products that were stable to prolonged digestion. Comparison of samples over the first 4 h showed that each "multimer-like" band in the digestion products had an electrophoretic mobility identical with that of one of the starting multimers. The protein moving at the rate of multimer 1 increased to a maximum at 8 h and was then degraded. The final products formed after 28 h were named by their elution order from a DEAE-Sephacel column. *S. aureus* V-8 protease fragment I was consistently observed as a faint band of high mobility. Fragment II was a major product with an electrophoretic mobility that was always less than that of vWF multimer 1. Its mobility varied, and in some composite gels, fragment II moved as fast as multimer 2 or 3, but occasionally it moved slower than the largest multimers. Fragment III was the second major product and had an intermediate mobility that was similar to fibrinogen. It is a triplet of decreasing intensity (bands IIIa, IIIb, and IIIc), and this triplet was referred to as fragment III. Three additional faint bands were clearly present on the original gels. One appeared between 0.5 and 4 h and had a mobility slightly faster than vWF multimer 1. The second was present between 2 and 16 h and moved between vWF multimer bands 2 and 3, while the third was a contaminant that migrated at the level of IgM and did not change during the digestion.

Laemmli gel electrophoresis of the digestion products gave a simpler pattern (Figure 2). The starting material contained a major band ($M_r$ 270K) and two very faint bands ($M_r$ 140K and 120K). The 270K band decreased with increased time of digestion and appeared to be transformed into two major (170K and 110K) and one minor (50K) product. Two-dimensional gel electrophoresis as well as electrophoresis of purified fragments clearly showed that fragments I–III could be assigned to the 50K, 110K, and 170K fragments, respectively. The faint bands of 140K and 120K rapidly decreased and were no longer visible after 4 h. Some polypeptides moved with the dye front and slowly increased. Lastly, a faint contaminant at 80K was not modified by digestion with V-8 protease. Western blots showed that two different monoclonal antibodies, previously shown to specifically react with the 270K and 140K bands (Chopek et al., 1986), also specifically reacted with the 110K fragment but not the 170K fragment.

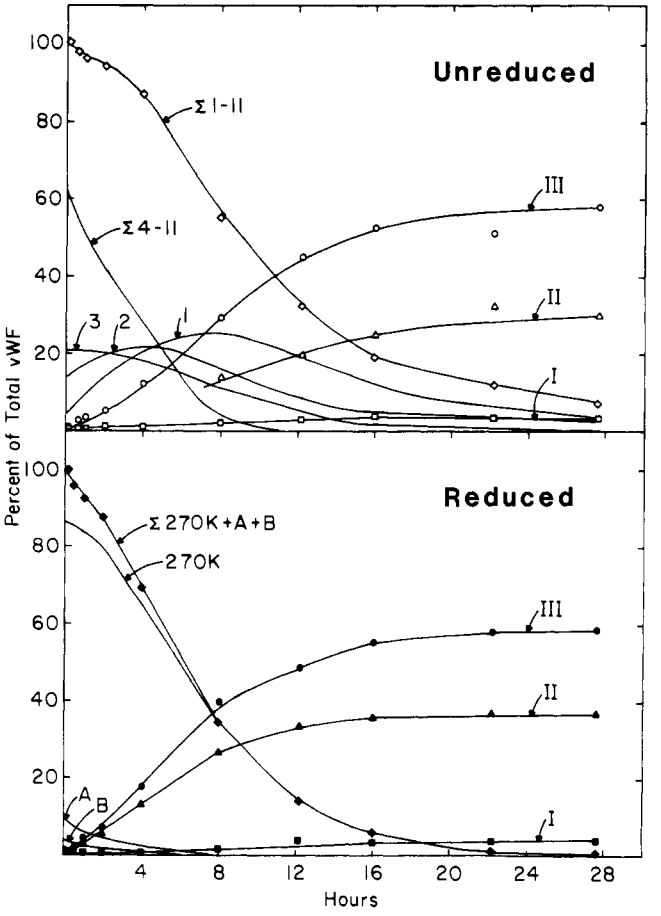


FIGURE 3: Time curve for digestion of vWF by *S. aureus* V-8 protease. Data were obtained by scanning the Coomassie-stained $NaDodSO_4$ pattern as described under Experimental Procedures. For each component, results are expressed as percent of the total vWF present at the time. (Top panel) Analysis without reduction. $\Sigma$1–11 represents the sum of the vWF bands 1–11; $\Sigma$4–11 is the sum of vWF bands 4–11; 1–3 refer to the vWF bands 1–3, respectively; I–III refer to fragments I–III. (bottom panel) Analysis after reduction. $\Sigma$270K+A+B refers to the sum of the three components (270K, 140K, and 120K) present in native vWF; 270K, A, and B refer to the same bands taken separately; I–III represent fragments I–III.

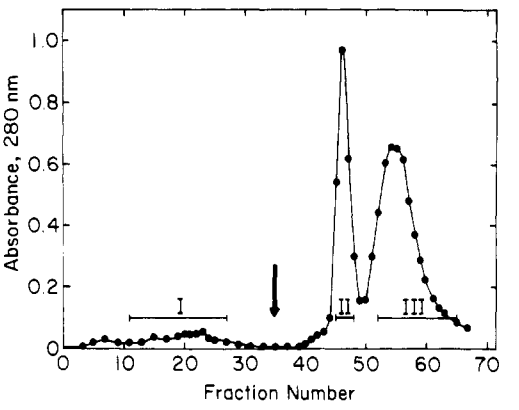


FIGURE 4: Ion exchange chromatography of *S. aureus* V-8 protease digest of vWF, vWF (40 mg) was digested by enzyme–Sepharose 2B complex for 20 h, treated with 10 mM DFP, dialyzed against 0.05 M ammonium acetate, pH 5.75, and applied to 20 mL of DEAE-Sephacel equilibrated in the same buffer. After being washed (140 mL), adsorbed fragments were eluted with 120 mL of a linear gradient from 0.05 to 0.5 M ammonium acetate, pH 5.75, and fractions (4 mL) were collected. Elution was followed by measuring the absorbance at 280 nm. The bars indicated pooled fractions and the arrow the start of the gradient.

The results obtained by quantitation of electrophoretic bands closely paralleled the qualitative estimates. Before reduction (Figure 3, top), the sum of vWF multimers 1–11 continuously decreased. Multimers from number 4 to 11 decreased individually and as a group. Multimer 3 remained constant over the first hour and then decreased. Multimers 2 and 1 increased, attained a maximum at 4 and 8 h, respectively, and then decreased. A clear relationship between the formation of fragments I–III and the disappearance of the starting vWF bands was noted. The curves for fragments II and III also indicate a parallel production of these species. The data from the first few hours showed a lag phase in the disappearance of vWF (multimers 1–11 and the 270K subunit) and in the production of fragments II and III. This lag phase was readily seen on a plot of logarithm of concentration vs. time. This plot also demonstrated that the disappearance of the 140K and 120K components could be approximated as a first-order process ($k = 9 \times 10^{-5}$ $s^{-1}$). A similar rate constant was found for the degradation of multimers 5–11 (taken as a group) and for the destruction of the 270K subunit after the lag phase.

Comparison of both curves in Figure 3 (unreduced and reduced) showed that the disappearance of the 270K subunit was faster than that of the sum of multimers 1–11. Similarly, the production of the 110K and 170K fragments II and III as estimated from the reduced samples was faster than the release of the free-terminal fragments II and III analyzed without reduction. These results suggest that during the course of digestion a significant amount of 110K and 170K species remains associated by disulfide bridges to the intact 270K subunit and result in a series of V-8 protease degraded vWF multimers that are electrophoretically similar to the native multimeric series. These differences were analyzed by plotting the degree of progression of each species (percent of its maximum) vs. the degree of digestion of the 270K subunit. This demonstrated that the rate of disappearance of vWF (multimers 1–11) was half that of the 270K subunit on a weight basis. Also, the rate of formation of fragments II and III (unreduced) was half to one-third the rate of the 270K digestion. At the end of the reaction, the formation rate of unreduced fragments II and III was twice as fast as the digestion of the 270K subunit. In contrast, when studied after reduction, the production of fragments II and III closely paralleled the disappearance of the 270K subunit, except for the latest times (22–28 h) when fragments II and III reached a maximum and then slightly decreased. The overall recovery of the digestion products as estimated by Coomassie blue staining and radioactivity in the electrophoretic bands was nearly quantitative. The final proportions of fragment II to fragment III, however, were about 2:3 by Coomassie blue staining and 1:1 by radioactivity.

*Purification and Characterization of vWF Digestion Products.* When the V-8 protease digest of vWF (20 h) was applied to DEAE-Sephacel in 0.05 M ammonium acetate, pH 5.8, a broad unadsorbed peak (I, fractions 5–30) was observed that accounted for 8% of the total absorbance (Figure 4). Elution with a linear gradient of ammonium acetate (0.05–0.5 M) at the same pH resolved a shoulder and two main peaks. Peak II eluted at 0.14–0.20 M ammonium acetate and represented 30% of the absorbance at 280 nm, whereas peak III eluted at 0.22–0.40 M and represented 61% of the absorbance. The total recovery was greater than 90%. Composite gel and Laemmli gel electrophoresis confirmed that these peaks were primarily fragments I–III, with a few minor contaminants (Figure 5). In particular, peak II contained a contaminant with mobility at the level of IgM on composite gel and $M_r$ 70K–80K after reduction. Determination of molecular weights by electrophoresis in $NaDodSO_4$–continuous and discontinuous polyacrylamide gel systems showed that fragment I had a $M_r$

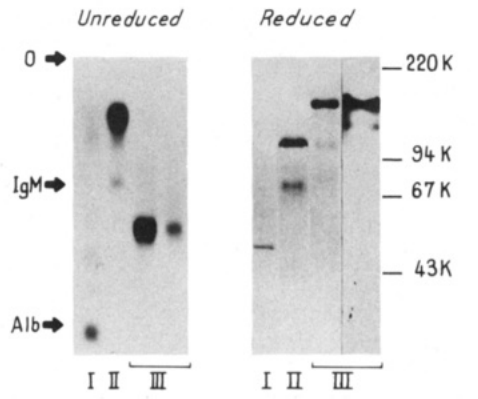


FIGURE 5: Electrophoretic analysis of purified fragments and protein bound to platelets. Fragments I–III were purified on DEAE-Sephacel as described under Experimental Procedures and Figure 4. $^{125}$I-Labeled fragment III was allowed to react with platelets in the presence of 1.2 mg/mL ristocetin, and after centrifugation through sucrose, the pellet was extracted with 6 M urea and 2 mM PMSF for 30 min at 40 °C. (Left panel) Autoradiograph after composite gel (Na-DodSO$_4$, urea, 1.6% polyacrylamide, 0.75% agarose) of labeled purified fragments and platelet extract. (Lane I) $^{125}$I-Labeled fragment I (~15000 cpm) with unlabeled fragment I (2.5 μg) as carrier; (lane II) $^{125}$I-labeled fragment II (~40000 cpm) with unlabeled fragment II (5 μg) as carrier; (lane III) (left) $^{125}$I-labeled fragment III (~30000 cpm) with unlabeled fragment III (4 μg) as carrier; (right) bound $^{125}$I-labeled fragment III (5000 cpm) extracted from the platelet pellet. The extract contained trace amounts of albumin, which served as carrier. Markers (left) show the positions of the origin, IgM, and albumin. (Right panel) Polyacrylamide gel (7.5%) electrophoresis pattern of purified fragments I–III and labeled platelet extract. (Lane I) Fragment I (3 μg); (lane II) fragment II (7 μg); (lane III) (left) fragment III (7 μg); (right) autoradiograph of bound $^{125}$I-labeled fragment III (5000 cpm) extracted from the platelet pellet and reduced. Markers (right) show the positions of the molecular weight standards and reduced fibronectin (220K).

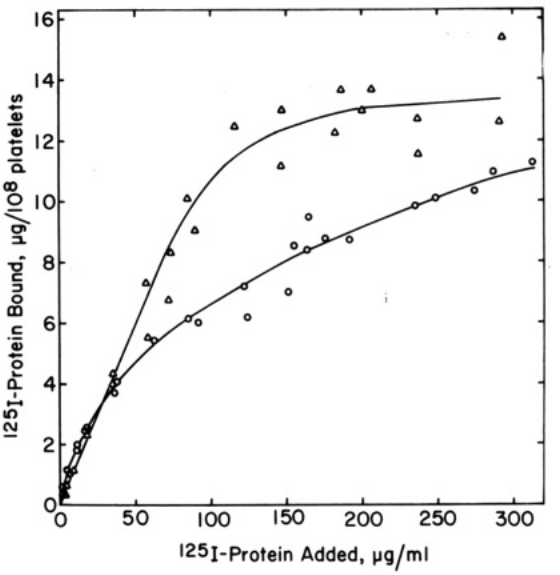


FIGURE 6: Specific binding of $^{125}$I-vWF and $^{125}$I-labeled fragment III to platelets in the presence of ristocetin. Different concentration of trace-labeled protein (0.005 μCi/μg) were incubated with formalin-fixed platelets ($10^8$/mL) in the presence of 1.2 mg/mL ristocetin. After 60 min at 20 °C, the platelets were centrifuged through sucrose, and associated radioactivity was counted. Nonspecific binding was subtracted from total binding. Each point represents the mean of duplicate determinations: (△) $^{125}$I-labeled fragment III; (○) $^{125}$I-vWF.

of 44K ± 3K unreduced and 52K ± 3K after reduction. Fragment II had $M_r$ 235K ± 40K unreduced and after reduction gave a band with $M_r$ 110K ± 10K. The major protein of the triplet fragment III (Figure 1, IIIa) had $M_r$ 315K ± 20K unreduced and 170K ± 10K after reduction. The second band of its triplet (Figure 1, IIIb) had $M_r$ 270K ± 20K reduced, and two-dimensional gel electrophoresis established that it was composed of the 170K chain associated with a fragment ($M_r$ 104K ± 4K) seen as a faint band on Figure 5 (right, III).

Because the products eluted from DEAE-Sephacel by an ammonium acetate gradient proved difficult to concentrate with high yield, a modified procedure was developed. At 0.075 M NaCl and pH 5.8, fragments I and II were not adsorbed to DEAE-Sephacel. These were readily precipitated with 40% $(NH_4)_2SO_4$ and separated from each other on Sephadex G-150, where fragment II eluted at 0.37 $V_e/V_t$ and fragment I at 0.57 $V_e/V_t$. Fragment III was eluted from the DEAE-Sephacel with 0.3 M NaCl, pH 5.8, as a sharp peak of concentrated protein.

*Amino-Terminal Sequence Analysis.* Fragments III, II, and I were then analyzed by Edman degradation to establish their location in the 270K subunit. In these experiments, 5 nmol of reduced and alkylated fragment III gave a sequence of Ser-Leu-Ser-Cys-Arg-Pro-Pro-Met-Val-Lys. This sequence is identical with that of the amino-terminal end of the 270K subunit (Hessel et al., 1984; Chopek et al., 1986). Fragment III contained about 1365 amino acid residues with a molecular weight of about 150000 for the carbohydrate-free fragment (Titani et al., 1986).

When 10 nmol of fragment II was subjected to Edman degradation, a sequence of Glu-Gln-Cys-Leu-Val-Pro-Asp-Ser-Ser-His was obtained. This sequence starts at about residue 1365 in the 270K subunit and is preceded by the sequence of Gln-Pro-Ile-Leu-Glu (Sadler et al., 1985; Titani et al., 1986). Thus, the principal cleavage site of V-8 protease occurs between an internal Glu–Glu peptide bond. This generates the amino-terminal fragment III and the carboxyl-terminal fragment II. The latter fragment contains 685 amino acid residues (Sadler et al., 1985; Titani et al., 1986), which is equivalent to a molecular weight of about 75000 for the polypeptide free of carbohydrate.

Edman degradation of 5 nmol of fragment I gave a sequence of Gly-Leu-Gln-Ile-Pro-X-Leu-Ser-Pro-Ala. This sequence starts at residue 911 in the 270K subunit and is preceded by the sequence of Val-Leu-Gln-Arg-Cys-Cys-Ser-Gly-Glu (Sadler et al., 1985; Titani et al., 1986). Accordingly, the minor cleavage site in vWF for V-8 protease occurs between an internal Glu–Gly peptide bond generating fragment I. This fragment composed of 454 amino acids originates from the carboxyl-terminal end of fragment III and has a molecular weight of about 51000 not including carbohydrate.

*Platelet Binding in the Presence of Ristocetin.* Preliminary experiments showed that fragment II was devoid of ristocetin cofactor activity, while fragment III had 12 units/mg as compared to 110 units/mg for the intact vWF. In the absence of ristocetin, platelet binding of $^{125}$I-labeled fragments I–III and $^{125}$I-vWF was less than 0.5% of the total radioactivity. This binding was not decreased when incubations were performed in the presence of 2.5 mg/mL unlabeled vWF. Thus, it was considered as nonspecific and was subtracted from total binding to estimate specific binding in all subsequent determinations. When estimated at very low ligand concentration (0.1 μg/mL), specific platelet binding in the presence of ristocetin expressed as percent ± SD of the total applied was 23 ± 5% for $^{125}$I-labeled fragment III ($n$ = 9) as compared with 53 ± 8% for $^{125}$I-vWF ($n$ = 26) and 20 ± 3% for partially reduced $^{125}$I-vWF ($n$ = 8). Control experiments demonstrated that in each case the only platelet-bound protein was fragment III (Figure 5), vWF, or partially reduced vWF. $^{125}$I-Labeled fragments I and II exhibited no specific binding.

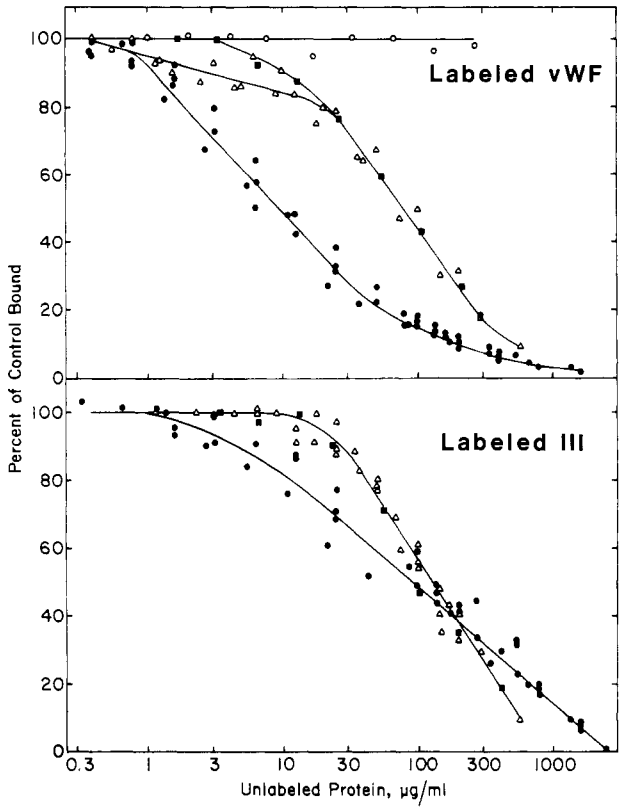


FIGURE 7: Competitive inhibition of $^{125}$I-vWF and $^{125}$I-labeled fragment III by unlabeled vWF, fragment II, fragment III, and partially reduced vWF. Formalin-fixed platelets ($10^8$ cells/mL) were incubated with a mixture of 0.1 μg/mL labeled protein and various concentrations of different unlabeled proteins in the presence of 1.2 mg/mL ristocetin. After 60 min at 22 °C, the platelets were centrifuged through sucrose, and the associated radioactivity was counted. The nonspecific binding was subtracted from total binding. Results are from several experiments. Each point is the mean of duplicate determination. (Top panel) $^{125}$I-vWF was incubated in the presence of (●) vWF (four experiments), (△) fragment III (three experiments), (○) fragment II (one experiment), and (■) partially reduced and alkylated vWF (one experiment). (Bottom panel) $^{125}$I-Labeled fragment III was incubated in the presence of (●) vWF (four experiments), (△) fragment III (four experiments), and (■) partially reduced and alkylated vWF (one experiment).

The concentration dependence of fragment III and vWF platelet binding in the presence of ristocetin was also examined (Figure 6). When studied between 0.1 and 1 μg/mL, a linear binding curve was obtained for both fragment III and vWF. Furthermore, vWF binding was 2.3 times greater than that of fragment III. At levels over 50 μg/mL, fragment III binding became higher than that of native vWF (Figure 6). These curves showed evidence of binding saturation; however, transformation of the data into Scatchard plots gave nonlinear curves for both proteins. The average amount of $^{125}$I-labeled protein bound at the highest concentrations with five different preparations was 16 ± 3 μg/$10^8$ platelets for fragment III and 12 ± 1 μg/$10^8$ platelets for vWF.

The identity of the platelet binding sites for $^{125}$I-labeled fragment III and $^{125}$I-vWF was further investigated by competitive inhibition experiments using a wide range of concentrations of native vWF, fragment II, fragment III, or partially reduced vWF (Figure 7). Since fragment I was only 4% of the total digest, binding inhibition studies were not performed with this fragment. With $^{125}$I-vWF, native vWF inhibited 50% of the binding at 9.4 μg/mL and more than 90% at 300 μg/mL (Figure 7, top). Under the same conditions, fragment III inhibited 50% of binding at 76 μg/mL and more than 90% at 600 μg/mL. Partially reduced vWF gave results very

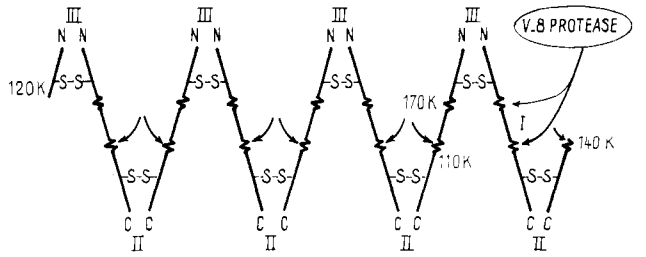


FIGURE 8: Proposed model for degradation of vWF by V-8 protease. Each vWF multimer consists of a linear arrangement of 270K subunits linked together by disulfide bridges. A multimer may end with 270K subunits or, as indicated in the figure, by either a 120K or a 140K polypeptide produced by proteolysis in vivo. V-8 protease acts by a primary cleavage (large arrow) in the 270K subunit between two glutamic acids located at residue 1365 from the N-terminus. This produces two complementary homodimers, including fragment II (the C-terminal portion, $M_r$ 2 × 110K) and fragment III (the N-terminal fragment, $M_r$ 2 × 170K). Fragment I ($M_r$ ~50K) results from a secondary cleavage by V-8 protease in the carboxyl-terminal region of fragment III (thin arrow) between a glutamic acid and a glycine at residue 911 from the N-terminus. This produces the 104K subunit from fragment III.

similar to fragment III. Both curves were superimposable except for the range of 1–30 μg/mL, where the fragment III appeared to inhibit a small amount of $^{125}$I-vWF binding in contrast to partially reduced vWF. Unlabeled fragment II did not inhibit $^{125}$I-vWF binding, even at 280 μg/mL (Figure 7, top). In similar experiments using $^{125}$I-labeled fragment III, unlabeled vWF inhibited 50% of binding at 90 μg/mL, and over 1.0 mg/mL was required for 90% inhibition (Figure 7, bottom). Unlabeled fragment III and partially reduced vWF again showed similar results, and each produced 50% inhibition at 130 μg/mL and greater than 90% inhibition at 580 μg/mL. The inhibition curves with unlabeled fragment III or partially reduced vWF were both steep (60-fold concentration range). In contrast, the inhibition curves for unlabeled vWF were less steep (300–1000-fold concentration range), and in the case of $^{125}$I-fragment III (Figure 7, bottom), this curve clearly crossed that obtained with unlabeled fragment III.

## Discussion

Limited proteolysis of vWF by *S. aureus* V-8 protease produced primarily two dimers that were called fragment II and fragment III (Figure 8). These fragments arise by proteolytic cleavage of the unique 270K vWF subunit to produce distinct, nonoverlapping portions of the 270K structure. Together they account for all of the initial 270K subunit. Amino-terminal sequence analysis demonstrated that fragment III originates from the amino-terminal portion of the 270K subunit and accounts for about 60% of the original subunit while fragment II originates from the carboxyl-terminal end and accounts for about 40% of the original molecule.

The kinetics of the digestion of vWF by V-8 protease showed that the decrease in starting components was counterbalanced by the production of fragments II and III and both products were formed at an equivalent rate, on a molar basis, throughout the reaction. In addition, the molecular weights of reduced fragment II (110K) and fragment III (170K) totaled that of the 270K subunit. These data indicate that V-8 protease produced one primary cleavage in the 270K subunit forming two fragments that were not linked by an intrachain disulfide bond(s).

It has been suggested that the electrophoretic heterogeneity of vWF multimers from plasma (Ruggeri & Zimmerman, 1981) or from factor VIII concentrate (Ohmori et al., 1982) was due to the assembly of vWF from nonidentical 270K subunits. Limited proteolysis of two 270K polypeptides with

nonidentical primary structures would produce three different patterns, i.e., an undigested 270K chain or large amounts of small peptides or two different pairs of complementary fragments. The present data do not support this model and are consistent with our other observations (Chopek et al., 1986) that vWF is assembled from a 270K subunit with a unique primary structure. Accordingly, the observed microheterogeneity (Ruggeri & Zimmerman, 1981; Ohmori et al., 1982) is probably related to either posttranslational modification, e.g., glycosylation or proteolysis, or to additional minor proteins that are assembled into the vWF complex.

Composite gels showed that during the first few hours of V-8 protease digestion vWF was transformed by integral jumps into smaller molecules with an electrophoretic mobility that was identical with that of one of the original multimers. Clearly, these newly formed products are different from the starting multimers, but their identical electrophoretic mobility suggests that they have similar structure and/or molecular weight. In also demonstrates that the protein mass that is removed during one jump is a multiple of the vWF basic assembly protomer, equivalent to the difference between multimer $n$ and multimer $n + 1$. Quantitation of composite gels showed that bands 3, 2, and 1 were in steady state at 0, 4, and 8 h, respectively, and bands 2 and 1 are intermediates. The protein in band 1 was the immediate precursor of the final dimers, fragments II and III. In addition, after the steady state of band 1 at 8 h, the rate of production of fragment II and III dimers was nearly the same as that of 270K cleavage, expressed on a molar basis. This suggests that the intermediate band 1 is composed of equimolar amounts of 270K, 170K, and 110K polypeptides. Its minimum molecular weight can be calculated from the sum of the equimolar components (540K) or from the sum of fragment II and III dimers (550K).

These data are consistent with the finding (Chopek et al., 1986) that vWF multimer 1 has a minimum molecular weight of 520K and is composed of equimolar amounts of three polypeptides with $M_r$ 270K, 140K, and 120K. In addition, the maximum yields of fragments II and III were 105% of the starting 270K subunit and 83% of starting 270K + 140K + 120K, which suggests that fragment II (110K) can also be formed from either the 140K or the 120K component. Furthermore, the Western blot results show that the 270K, 140K, and 110K polypeptides share at least two epitopes and suggest that fragment II (110K) can be formed by V-8 proteolysis of the 140K polypeptide. The analogy between the fragments obtained by V-8 protease digestion (170K and 110K) and the components found in vWF multimers (140K and 120K) and the analogy between the composition of the V-8 intermediate band 1 and vWF multimer 1 suggest that there is at least one protease-sensitive region within the 270K subunit of vWF multimers and that the 140K and 120K vWF components arise by proteolysis within this region.

The present data are consistent with an alternating linkage by disulfide bonds of the amino- and carboxyl-terminal ends of the 270K subunits, as recently suggested by Hamilton et al. (1985). With this proposed structure, the release of one fragment II or fragment III dimer would require the cleavage of two or more adjacent 270K subunits. Assuming that the 140K and 120K vWF components are also derived from proteolysis of the 270K subunit, they may be the free ends of such multimeric structure (Chopek et al., 1986). However, this model does not explain why the V-8 protease digestion produces an unreduced fragment II with a slower mobility than the larger fragment III. Also, it does not account for all the predicted intermediates generated from vWF by the V-8 protease. Thus, further studies are required to refine this model.

Platelet binding assays performed with $^{125}$I-labeled purified fragments and $^{125}$I-vWF in the presence of ristocetin confirm our previous findings with the whole *S. aureus* V-8 digest (Chopek et al., 1986). Fragment I and fragment II did not bind to these platelets, whereas fragment III and vWF readily bound. In their study of $^{125}$I-vWF binding to platelets in the presence of ristocetin, Gralnick et al. (1981) observed nonlinear Scatchard plots and reported about 3000 high-affinity and 25 000 low-affinity binding sites per platelet. Kao et al. (1979) also used Scatchard plots and estimated 31 000 molecules bound per platelet. In these experiments, platelet binding, at low protein concentrations, was linear and higher for vWF than for fragment III (53% vs. 23%); however, the maximal binding of fragment III was higher than observed for native vWF (16 vs. 12 $\mu g/10^8$ platelets). This represents maximal binding of 300 000 molecules/platelet for fragment III and 15 500 or 31 000 molecules/platelet for vWF, assuming weight-average molecular weights of either $2.15 \times 10^6$ or $4.3 \times 10^6$ daltons. The values for vWF binding are within the range determined by others; however, fragment III binding is 10-fold higher than anticipated. Thus, although native vWF has a higher apparent binding constant than fragment III, these data suggest that many more fragment III molecules can bind to each platelet.

The competitive inhibition data are complex, but reveal several interesting features. Ristocetin-induced platelet binding of labeled vWF or fragment III can be completely inhibited by unlabeled native vWF, fragment III, or partially, reduced vWF. Thus, native vWF, fragment III, and partially reduced vWF must all share the same binding sites on platelet membranes. The overall binding constant of vWF for platelets appears to be higher than that of fragment III or the vWF dimer. This has been previously shown for partially reduced vWF (Gralnick et al., 1981; Ohmori et al., 1982) and for smaller multimers (Martin et al., 1981; Chopek et al., 1986). The broad inhibition curves obtained with native vWF are probably a reflection of the heterogeneity of vWF multimer size and apparent binding constant for platelets. The binding inhibition curves for fragment III and for partially reduced vWF are very similar. Since reduction and proteolysis presumably occur at different parts of the molecule, we conclude that both fragment III and vWF dimer have discrete, functionally intact and similar binding site(s). These data strongly support our previous proposal that vWF is a multivalent protein and consists of a relatively linear array of identical binding sites. This hypothesis attributes the higher apparent binding constant of high molecular weight vWF multimers to their larger size and ability to interact with more than one platelet binding site.

There are at least three possibilities to explain the unexpected observations that although both vWF and fragment III compete for the same platelet binding site(s), fragment III can bind far more molecules than native vWF. First, if larger multimers were able to interact with multiple platelet binding sites, this might give a higher apparent binding constant for these multimers as well as occupy more platelet sites per molecule of vWF. Second, there may be two different ristocetin-induced binding sites on the platelet membrane, a small number with high apparent affinity for high molecular weight vWF multimers and a large number with lower apparent affinity for all vWF multimers; however, both of these sites may have similar affinity for fragment III. Lastly, there may be only one type of platelet binding site, but this type may have a different steric accessibility to native vWF multimers; access

to binding sites may be limited for larger vWF multimers, whereas fragment III, vWF dimer, or smaller multimers may interact with all sites equally.

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