Proteolytic Studies on the Structure of Bovine von Willebrand Factor†

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ABSTRACT: Bovine von Willebrand factor (vWF) was digested with protease I (P-I), a metalloprotease isolated from rattlesnake venom. Digestion of vWF for 24 h with P-I yielded a terminal digest consisting of an equimolar mixture of two major fragments (apparent M_r 250K and 200K). The 250-kilodalton (kDa) fragment consists of a 125-kDa chain from one subunit and a 45- and 78-kDa polypeptide chain from an adjacent subunit. The 200-kDa fragment consists of a 97-kDa chain from one subunit and a 35- and 61-kDa polypeptide chain from an adjacent subunit. The 200-kDa fragment binds to heparin, and the heparin binding domain is located on the 97-kDa polypeptide chain. This fragment also competes with labeled, native vWF for binding to formalin-fixed human platelets, with an IC_{50} of 12.5 μg/mL (65 nM). However, native vWF has an IC_{50} of 2.5 μg/mL, indicating that the affinity of the 200-kDa fragment for platelets is approximately one-fifth that of vWF. The 200-kDa fragment agglutinates platelets, but its agglutinating ability is only 5% that of the native molecule. Only the 200-kDa fragment is recognized by monoclonal antibodies 2 and H-9, which are directed against vWF and inhibit vWF binding to platelet glycoprotein Ib (GPIb). Immunological studies, using nine monoclonal antibodies directed against vWF, and the demonstration that the heparin and GPIb binding domains are located on only one fragment suggest that the two fragments are composed of different regions of the vWF subunit. Analysis of the P-I cleavage pattern suggests that all vWF subunits are not cleaved in the same fashion. The first cleavage on half of the subunits generates the 45-kDa terminal and 175-kDa intermediate digest products. The 175-kDa chain is again cleaved, producing the 97- and 78-kDa terminal polypeptide chains. However, the first cleavage of the other subunits generates the 35-kDa terminal and the 186-kDa intermediate digest product, which upon cleavage produces the 125- and 61-kDa terminal polypeptide chains. Immunological data support the asymmetric cleavage pattern. An epitope for a monoclonal antibody is present on both the 186- and 175-kDa intermediate digest products but is only found on one terminal digest fragment, the 78-kDa polypeptide chain, suggesting that the 186- and 175-kDa polypeptides are cleaved at different sites. Epitope mapping of the fragments also indicates that they share a common region of the subunit. The presence of an overlapping region is possible if adjacent fragments are not cleaved in the same fashion. The basic protomer of vWF is a dimer of 230-kDa polypeptide chains. Analysis of proteolytic patterns of iodinated vWF suggests that physical differences exist between the two chains of a dimer. One of the chains contains a region which is highly susceptible to iodination; this region is located on the 175-kDa intermediate and 78-kDa terminal digest products. A structural model of bovine vWF has been developed in which the subunits are linked by disulfide bonds that alternate between two carboxyl-terminal and two amino-terminal regions in a head-to-head/tail-to-tail fashion. The proteolytic cleavage patterns and preferential iodination of certain subunits indicate that structural differences exist between adjacent subunits.

A von Willebrand factor (vWF) is a high molecular weight, oligomeric, plasma glycoprotein which is required for platelets to recognize damaged endothelial surfaces (Baumgartner et al., 1980). It can act as a bridge between platelet membranes and subendothelial tissue at sites of endothelial damage (Sakariassen et al., 1979). In the presence of ristocetin, vWF binds to glycoprotein Ib (Nurden & Caen, 1975). It also binds to the glycoprotein IIb/IIIa complex on platelets stimulated with thrombin or ADP (Fujimoto et al., 1982; Ruggeri et al., 1982). The major platelet receptor for vWF is thought to be glycoprotein Ib (GPIb). The importance of this interaction is suggested by the prolonged bleeding times seen in patients with Bernard–Soulier syndrome, a disorder characterized by the lack of functional GPIb (Jamieson et al., 1979). These patients have long bleeding times associated with defective platelet adhesion. Bovine vWF binds to GPIb directly, without ristocetin (Coller et al., 1982). Evidence suggests that bovine vWF and human vWF bind to GPIb in a similar manner. For instance, agglutination of human platelets by bovine and human vWF is inhibited by antibodies to GPIb (Tobelem et al., 1976). Platelets from individuals with Bernard–Soulier syndrome are not agglutinated by bovine or human vWF (Caen et al., 1976). Also, bovine vWF and human vWF bind to human formalin-fixed platelets in a reversible manner and at the same site (Suzuki et al., 1980).

vWF has been shown to bind to many different components of the subendothelial matrix. The binding of vWF to both interstitial and basement membrane collagens has been demonstrated (Santoro, 1981; Santoro & Cowen, 1982; Morton et al., 1983). vWF has also been shown to bind to microfibrils (Fauvel et al., 1983) and the glycosaminoglycan heparin (Suzuki et al., 1979; Amrani et al., 1982).

vWF is composed of subunits which have an apparent molecular weight of 230K on sodium dodecyl sulfate (Na-$DodSO_4$)–polyacrylamide gels (Counts et al., 1978). The monomers are covalently linked by disulfide bonds to form

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heterogeneous oligomers in plasma, ranging from 1×10^6 to 20×10^6 (Ruggeri & Zimmerman, 1980a), with the majority of the vWF protein existing as higher molecular weight oligomers (Ruggeri & Zimmerman, 1980b). A vWF protomer consists of two polypeptide chains which are covalently linked by one or more disulfide bonds. These dimers, in turn, are disulfide linked to form higher molecular weight oligomers (Counts et al., 1978). The interchain disulfide bounds that form a dimer are quite stable, since treatment of native vWF with mild reducing agents causes a stepwise decrease in the higher molecular weight oligomers and produces primarily vWF dimers. Ohmori et al. (1982) have shown that vWF appears in the electron microscope as strands of various lengths, consistent with the size heterogeneity shown by Na-DodSO$_4$–agarose gel electrophoresis. Fowler et al. (1985) have shown that vWF multimers, which appear as flexible strands varying in length up to 2 μm, consist of dimeric subunits (protomers) polymerized linearly in an end-to-end fashion through disulfide bonds. The electron micrographs suggest that dimers are approximately 100–120 nm long and consist of two large globular domains connected by a small central node by two flexible domains.

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 human vWF is composed of a single chain containing 2050 amino acids and 22 carbohydrate chains. This yields a subunit of about 270 kilodaltons (kDa) for human vWF.

Recently, several studies have been undertaken to isolate and characterize the regions of the vWF molecule that interact with specific receptors on the platelet membrane and components of the subendothelium. Martin et al. (1980) found that prolonged digestion of native vWF did not lead to complete loss of ristocetin-induced platelet agglutinating activity, even though no trace of intact vWF subunit could be detected by gel electrophoresis. They isolated a tryptic fragment (M_r 116K) which accounted for this residual activity. A similar tryptic fragment is recognized by a monoclonal antibody that inhibits vWF-mediated platelet agglutination (Sixma et al., 1984). We previously reported the isolation and characterization of a plasmic fragment of bovine vWF (M_r 250K) which binds directly to GPIb on human platelets (Mascelli et al., 1986). Girma et al. (1986) found that staphylococcal V-8 protease cleaves native vWF into two major fragments that are not connected by disulfide bonds. One fragment (designated Sp III, M_r 315K) is a disulfide-linked homodimer of two 170-kDa polypeptides from the amino terminus; it binds to GPIb in the presence of ristocetin. The other fragment (designated Sp II, M_r 235K) is also a disulfide-linked homodimer of two 100-kDa polypeptide chains from the carboxyl-terminal end of the protein. It binds to GPIIb/IIa on thrombin- and ADP-stimulated platelets. These data indicate that the vWF subunits are linked by disulfide bonds that alternate between two carboxyl-terminal and two amino-terminal regions of the molecule in a head-to-head/tail-to-tail manner. Fretto et al. (1986) studied electron micrographs of the Sp II and Sp III fragments. They revealed that the vWF multimers are cleaved by V-8 protease where the flexible rod domains join the large globular domains. The Sp III fragment is a disulfide-linked homodimer of the large globular domains of two subunits. The Sp II fragment is a disulfide-linked homodimer of the flexible rod domains of two subunits.

This paper describes the isolation and characterization of the major proteolytic products of bovine vWF which has been digested with protease I, a metalloprotease found in the venom of the Western Diamondback rattlesnake. The fragments have been analyzed for their ability to bind to GPIb and heparin. The results suggest that bovine vWF is assembled in a manner similar to human vWF, with the subunits arranged in a head-to-head/tail-to-tail fashion. However, cleavage patterns, chemical modification studies, and approximate localization of monoclonal antibody epitopes indicate that the two subunits of vWF in a dimer can be distinguished from one another.

Materials and Methods

Reagents. Materials were purchased from the following companies: nitrocellulose filter paper (0.45 μm) from Millipore Corp. (Bedford, MA); Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) from Pierce Chemical Co. (Rockford, IL); Sepharose CL-6B from Pharmacia Fine Chemicals (Sweden); goat anti-mouse IgG and normal goat serum from Pel-Freez Biologicals (Rogers, AR); acrylamide and *N*,*N*-methylenebis(acrylamide) from Bio-Rad (Richmond, CA); X-ray film (type XAR-5) from Eastman Kodak (Rochester, NY). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO). Protease I, purified from the venom of the Western Diamondback rattlesnake (Pandya & Budzynski, 1984), was a gift from Dr. Andrei Budzynski, Thrombosis Research Center, Temple University. The monoclonal antibody directed against human factor XII was a gift from Dr. Robin Pixley, Thrombosis Research Center, Temple University.

Buffers. Phosphate saline buffer (PBS) contained 0.01 M phosphate, 0.15 M NaCl, and 0.02% NaN_3, pH 6.5. Immunoblotting buffer was 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 147 mM NaCl, and 3.1 mM NaN_3, pH 7.2, containing 0.1% bovine serum albumin (BSA) and 0.005% Tween 20.

Gel Electrophoresis. NaDodSO$_4$–polyacrylamide gels (7.5%) were prepared by the procedure of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 as described by Fairbanks et al. (1971).

Densitometric Scanning. Coomassie Blue stained NaDodSO$_4$–polyacrylamide gels and autoradiograms of gels were scanned on a Hoeffer GS-300, single-beam densitometer (Hoeffer Scientific Instruments, San Francisco, CA). Gels were scanned prior to drying or after they were dried between two sheets of cellophane. The quantity of each fragment was determined by measuring the relative areas of the corresponding peaks.

Protein Determinations. Protein concentrations were determined by the procedure of Lowry et al. (1951) using bovine serum albumin as a standard or from the absorbance of the protein at 280 nm, using a value of 12 for $E^{1\%}_{280nm}$. The A_{280nm} was corrected for light scattering by subtracting 1.7 times the absorbance at 320 nm (Cantor & Schimmel, 1980).

Purification of vWF. Bovine vWF was purified as described previously by Mascelli et al. (1986). Reduced NaDodSO$_4$–polyacrylamide gel electrophoresis of the concentrated protein showed a single band with an apparent molecular weight of 230K.

Iodination Procedures. (1) Iodination of vWF and Goat Anti-Mouse IgG. These proteins were iodinated by the Iodogen method (Markwell & Fox, 1978) as previously described (Mascelli et al., 1986). All radioiodinated preparations of vWF used for experiments had 0.5–1.0 mol of iodine incorporated per subunit and had a specific activity of approximately 200–400 μCi/mg of vWF. Previous studies (Kirby, 1982) have shown that up to two atoms of iodine can be incorporated per subunit without significant loss of either platelet binding or agglutinating activity.

(*2*) *Iodination of Bolton–Hunter Reagent.* Iodinated Bolton–Hunter reagent (^{125}I-BHR) was prepared by adding 5 μL of an 8.4 mg/mL solution of the reagent *N*-succinimidyl 3-(4-hydroxyphenyl)propionate in dry dimethylformamide to a septum-capped glass reaction vial, followed by 10 μL of a 40 mM solution of sodium iodide containing 200 μCi of Na^{125}I. The reaction was immediately initiated by the addition of 50 μL of Chloramine T (5 mg/mL) in 0.25 M sodium phosphate, pH 7.5, followed within 20–30 s by 50 μL of sodium metabisulfite (6 mg/mL in 0.05 M sodium phosphate, pH 7.5) and 0.5 mL of benzene. The benzene extracts were evaporated under a stream of nitrogen and stored in a desiccator over P_2O_5. The purity of the ^{125}I-BHR was established by thin-layer chromatography on silica gel plates as described by Bolton and Hunter (1973), using standards of unlabeled mono- and diiodo reagent. The reagent used for experiments was more than 90% diiodo-BHR and was essentially free of hydrolysis products. The concentration of the reagent was determined from its radioactivity and the specific activity of the ^{125}I-iodide used in synthesis, assuming two atoms of iodine were incorporated per BHR.

(*3*) *Labeling of vWF with ^{125}I-BHR.* vWF was labeled with ^{125}I-BHR as previously described (Silverman et al., 1987). ^{125}I-BHR was dissolved in benzene and dried on the sides of 1.5-mL Eppendorf tubes. Solutions of vWF in 0.01 M borate/0.15 M NaCl buffer, pH 8.5, were added to the tubes and incubated for 1 h at room temperature with occasional mixing. The reaction had generally ceased after 30 min, due to hydrolysis of unreacted reagent. Under these conditions, between 40% and 60% of the reagent was incorporated into the protein. Incorporation of the reagent into vWF was measured by dilution of aliquots into 0.5 mL of 0.02 M sodium acetate, 0.15 M NaCl, and 1 mM NaI, pH 5.0, containing 200 μg/mL BSA, followed immediately by 0.5 mL of cold 20% trichloroacetic acid. Parallel experiments showed that the low pH and prior hydrolysis of most of the ^{125}I-BHR prevented reaction of the BSA with the reagent. After 1 h on ice, precipitates were collected by centrifugation and washed with 1 mL of cold 10% trichloroacetic acid, and then the pellets and supernatants were counted to determine the extent of incorporation of the radioactive reagent into vWF.

Digestion of vWF with Protease I. Protease I was added at a concentration of 1 μg of protease per 100 μg of vWF at 37 °C in PBS. Digestion was stopped by the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of 2 mM.

Preparation of Formalin-Fixed Platelets. Platelet concentrates which were less than 24 h old were a gift of the Penn-Jersey Regional Red Cross Blood Program. The platelet concentrates were formalin-fixed as previously described (Kirby, 1982). The formalin-fixed platelets were stored at −90 °C in PBS containing 25 mg/mL BSA.

Binding Studies. Frozen, formalin-fixed platelets were thawed, kept at room temperature for the 1–2 h needed for most experiments, and then prewarmed to 37 °C 10 min prior to doing binding studies. All binding analyses were performed in 1.5-mL Eppendorf tubes in the presence of BSA (10 mg/mL) to decrease nonspecific binding. The proteins were added to the tubes, and the volume was adjusted to 300 μL with PBS containing 1% BSA. A suspension of formalin-fixed platelets (700 μL) was added to give a final concentration of 2.2×10^8 platelets/mL. Tubes were inverted to mix the contents and then incubated at 37 °C for 6 min without stirring. The platelets were pelleted by centrifugation for 2 min at 12000*g* in an Eppendorf Model 3200 centrifuge equipped with a swinging-bucket rotor. Supernatants were removed with siliconized Pasteur pipets that had been drawn out to fine capillaries. The tips of the centrifuge tubes were cut off with a razor blade, and supernatants and pellets were counted for ^{125}I γ emission (Nuclear Enterprises Model 1600 τ counter). Formalin fixation of the platelets and the absence of divalent calcium ions in the system ensure that the binding observed is to the GPIb-associated receptor, rather than to the GPIIb/IIIa receptor.

Platelet Agglutination Studies. Agglutination of formalin-fixed, human platelets was measured in an aggregometer (Payton Instruments, Buffalo, NY) at 37 °C, with a stirring speed of 1400 rpm. Platelets were diluted to approximately 2.5×10^8 platelets/mL in PBS, and 700 μL was put into a 6.4-mm inner diameter cuvette. After being prewarmed at 37 °C for several minutes, the cuvette was transferred to the aggregometer, and an aliquot of protein solution (10–100 μL) was added. The agglutination rate was measured as the slope of the steepest part of the agglutination trace. A standard curve was generated by measuring the agglutination rate with different concentrations of vWF. A platelet agglutinating activity of 1.0 was defined as equivalent to the agglutination rate produced by 1 μg of vWF.

Immunoblotting Studies. Monoclonal antibodies directed against bovine vWF were raised, purified, and characterized as previously described (Mascelli et al., 1986). Monoclonal antibody H-9, directed against human vWF, was a generous gift from Dr. Dominique Meyer, Institute de Pathologie Cellulaire, Hôpital de Bicêtre. Monoclonal antibody H-9 inhibits both binding of vWF to the GPIb receptor and vWF-mediated platelet adhesion to subendothelium (Meyer et al., 1984).

Proteins were blotted onto nitrocellulose filters in one of two ways. Either protein solutions (20 μL) were spotted directly with a Hamilton syringe or, alternatively, the proteins were first electrophoresed on 7.5% NaDodSO$_4$–polyacrylamide gels and then transferred to nitrocellulose paper by electrophoresis (Towbin et al., 1979) at 100 mA (30 V) for 24 h at room temperature. Nonreacted sites on the filter paper were blocked by incubating with PBS containing 3% BSA for 1 h at room temperature with gentle shaking. Individual papers were incubated first with one of the monoclonal antibodies and then with ^{125}I-labeled goat anti-mouse IgG (approximately 1×10^6 cpm). The antibody incubations were 24 h in length at 4 °C. The antibodies were diluted in immunoblotting buffer. The papers were washed between antibody incubations with three 0.5-h washes of immunoblotting buffer at room temperature.

Gel Chromatography. Gel filtration columns (Sepharose CL-6B) were standardized with the following globular proteins: bovine IgM (M_r 1×10^6), bovine thyroglobulin (M_r 670K), bovine ferritin (M_r 450K), and human IgG (M_r 150K).

Results

Analysis of Protease I Generated Fragments of vWF by NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. vWF was incubated with P-I, and at intervals, aliquots were removed, and the digestion was stopped with EDTA. Samples were analyzed on NaDodSO$_4$–polyacrylamide gels (Figure 1). Native vWF (lane 1) was too large to enter the stacking gel. However, as the time of proteolysis increased, vWF was cleaved into fragments small enough to enter the gel. After 24 h of digestion, P-I had cleaved vWF into two major fragments of M_r 250K and 200K. Densitometric scanning of the lane containing the 24-h sample indicated that the two fragments were present in equimolar ratios. Addition of more P-I at 24 h, with incubation for an additional 24 h, did not result

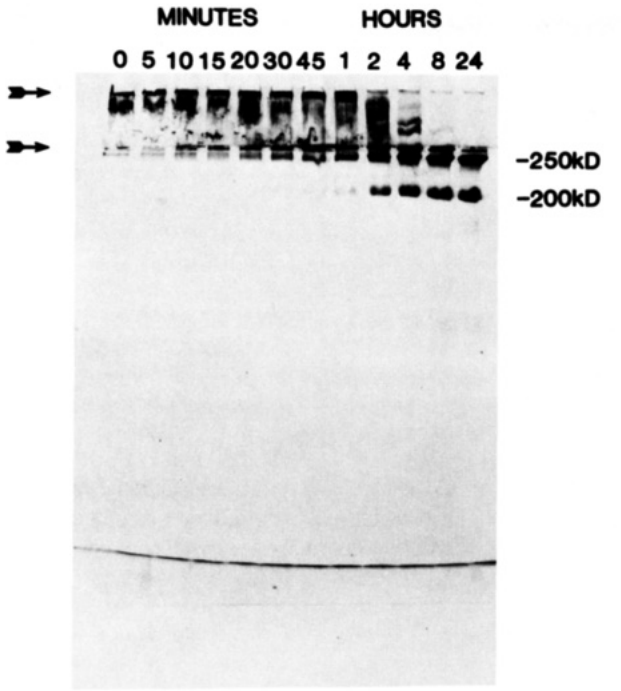

FIGURE 1: Time course of P-I digestion of bovine vWF, analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. vWF (0.45 mg/mL) was incubated with protease I at 37 °C. At the indicated times, aliquots were removed and diluted with an equal volume of $NaDodSO_4$ diluent buffer. Samples were electrophoresed on a 7.5% $NaDodSO_4$–polyacrylamide gel. The gel was stained with Coomassie Blue. The arrows represent the tops of the stacking and the running gel, respectively.

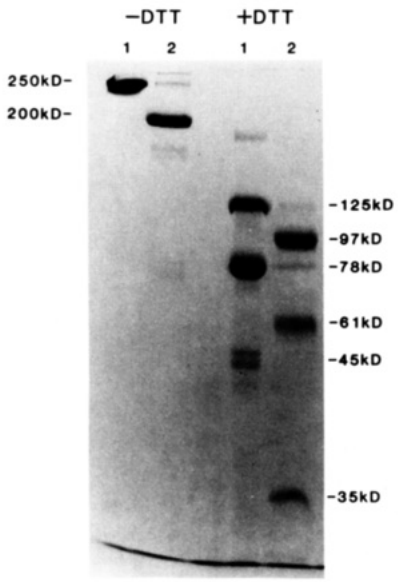

FIGURE 2: Purification of the 250- and 200-kDa fragments by heparin–agarose chromatography. vWF (900 μg) was incubated with P-I for 24 h. The protein was applied to a 1-mL heparin–agarose column equilibrated in PBS. Proteins were eluted first with PBS (fraction 1) and then with 0.01 M phosphate–1.0 M NaCl buffer, pH 7.0 (fraction 2). These fractions were electrophoresed on a 7.5% $NaDodSO_4$–polyacrylamide gel with or without prior reduction with 0.5% dithiothreitol (DTT).

in further cleavage, indicating that the two fragments generated after 24 h of proteolysis were terminal digestion products of vWF. vWF, 250-, and 200-kDa fragments were assayed for neutral sugars by a phenol sulfuric acid assay. Native vWF was found to contain approximately 11% carbohydrate (micrograms of sugar per microgram of protein), in agreement with the amount previously determined by

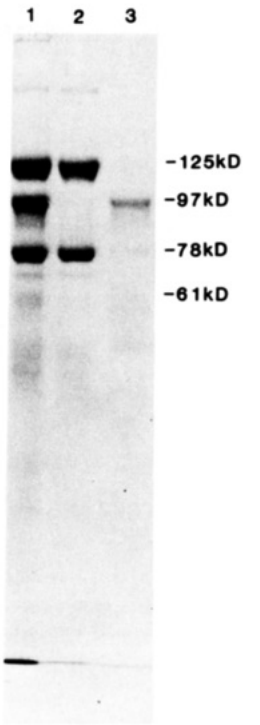

FIGURE 3: Identification of the polypeptide chain which binds to heparin. vWF (600 μg) was digested with P-I for 24 h. The digested protein was incubated with 20 mM β-mercaptoethanol for 1 h at 37 °C in 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, containing 8 M urea and then with 40 mM iodoacetamide for 2 h at 37 °C. The protein was applied to a 2-mL heparin–agarose column equilibrated with Tris–urea buffer. Polypeptides were eluted first with Tris–urea buffer and then with 0.01 M phosphate–1.0 M NaCl, pH 7.0. Peak fractions of each eluent were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue. Lane 1, precolumn sample (reduced and alkylated terminal P-I digest of vWF); lane 2, polypeptides which eluted in Tris–urea buffer; lane 3, polypeptide which eluted in 1.0 M NaCl buffer.

Schmer et al. (1972). The 200-kDa fragment contained slightly more neutral sugar (12%) than the 250-kDa fragment (9%).

Identification of the Fragment That Binds to Heparin. It was demonstrated by Bockenstedt et al. (1986) that human vWF binds to heparin. To identify which fragment of bovine vWF binds to heparin, vWF which had been incubated with P-I for 24 h was applied to a column containing heparin–agarose, and the fractions were analyzed on $NaDodSO_4$–polyacrylamide gels (Figure 2). The 200-kDa fragment, but not the 250-kDa fragment, was retained and could be eluted with 1 M NaCl.

When the purified fragments were reduced prior to electrophoresis, it was revealed that both fragments were composed of three disulfide-bonded polypeptide chains; the 250-kDa fragment was composed of a 125-kDa, a 78-kDa, and a 45-kDa polypeptide chain, and the 200-kDa fragment was composed of a 97-kDa, a 61-kDa, and a 35-kDa polypeptide chain.

In order to determine which of the polypeptide chains of the 200-kDa fragment contained the heparin binding site, a terminal P-I digest of vWF was reduced and alkylated prior to chromatography on a heparin–agarose column (Figure 3). The 125- and 78-kDa polypeptides, which are part of the 250-kDa fragment, were not retained, but the 97-kDa polypeptide of the 200-kDa fragment was retained and subsequently eluted. The Coomassie staining of the 61-, 45-, and 35-kDa polypeptides was too faint to determine whether or not they were retained on the column.

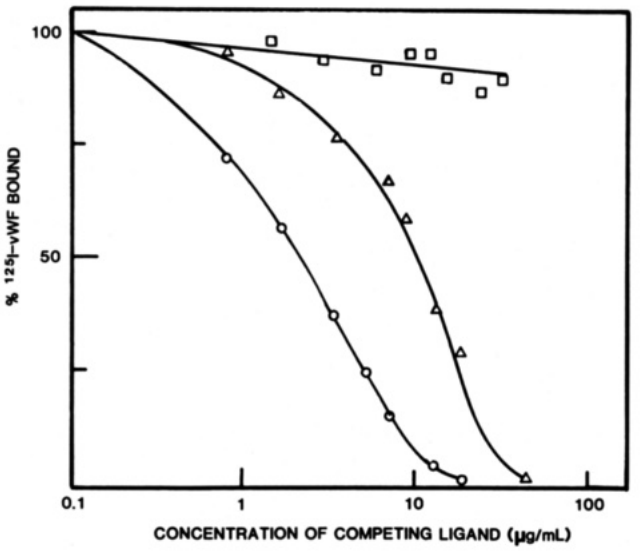

FIGURE 4: Analysis of P-I digest fragments for platelet binding ability. ^{125}I-vWF (100 ng) was incubated with human formalin-fixed platelets (2.2×10^8 platelets/mL) and different concentrations of either native vWF (○), 250-kDa fragment (□), or 200-kDa fragment (△) for 6 min at 37 °C. The samples were centrifuged, and the platelet pellets and supernatants were counted to determine the amount of ^{125}I-vWF bound to platelets. Each point represents the mean of duplicate determinations.

Platelet Binding and Agglutination Studies. To determine whether the GPIb binding domain was retained after digestion, the 250- and 200-kDa fragments were compared with native vWF for their ability to compete with ^{125}I-vWF for binding to the GPIb receptor on formalin-fixed platelets (Figure 4). A vWF concentration of approximately 2.2 μg/mL (13 nM) was required to inhibit binding of ^{125}I-vWF by 50%. The 200-kDa fragment also inhibited ^{125}I-vWF binding, but approximately 5 times more of the 200-kDa fragment was required to inhibit binding of ^{125}I-vWF by 50%. In contrast, the 250-kDa fragment did not compete with ^{125}I-vWF for binding to platelets.

Since the 200-kDa fragment bound to platelet GPIb, it was tested for its ability to agglutinate platelets; 200-kDa fragment purified by heparin–agarose chromatography was not satisfactory to perform the experiment, since NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 2) revealed that it contained a small amount of higher molecular weight contaminants, which were incompletely fragmented vWF. In order to obtain 200-kDa fragment free of contaminants, a 24-h P-I digest of vWF was chromatographed over a molecular exclusion column (Figure 5), and peak fractions were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 5B). The first peak, which eluted at the void volume, probably consisted of partially digested vWF. When an aliquot of this peak was analyzed by gel electrophoresis, no bands were visualized by Coomassie staining, probably because the protein was too dilute and represented a series of partially digested fragments. The second peak consisted of the 250-kDa fragment. The third peak contained primarily the 200-kDa fragment which was slightly contaminated with the 250-kDa fragment. The last peak eluted at the inclusion volume of the column and did not contain protein but represented a UV-absorbing contaminant which was present in the poly(ethylene glycol) used to concentrate the vWF. Aliquots of each fraction were assayed for their ability to agglutinate platelets. A small amount of platelet agglutinating activity was detected in the first peak, which contained partially digested vWF. The majority of the activity, however, eluted in the same position as the 200-kDa fragment. The platelet agglutinating activity of the 200-kDa fragment was approximately 5% that of native vWF. The 250-kDa fragment had no detectable platelet agglutinating activity.

The molecular exclusion column was calibrated with four globular proteins: human IgM, bovine thyroglobulin, bovine ferritin, and bovine IgG. The 250-kDa fragment eluted at a Stokes radius equivalent to a molecular weight of 1×10^6 for a globular protein, implying either that the shape of this fragment is very asymmetric or that it self-associates to form noncovalent oligomers. The 200-kDa fragment, however, eluted at M_r 200K, suggesting that the fragment is approximately globular in nature and does not form noncovalent aggregates. Since it can agglutinate platelets, these data also suggest that there are two GPIb binding domains on each fragment.

Polypeptide Composition of a Protease I Digest of vWF. Reduced samples of vWF which had been digested with P-I for various times were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis in order to identify the polypeptide chains of vWF which comprise a digest (Figure 6). In this experiment, the vWF preparation contained a contaminant which electrophoresed as a triplet band of M_r 55K. The contaminant was most likely fibrinogen or fibrin polymer which had been partially proteolyzed during purification of vWF. The di-

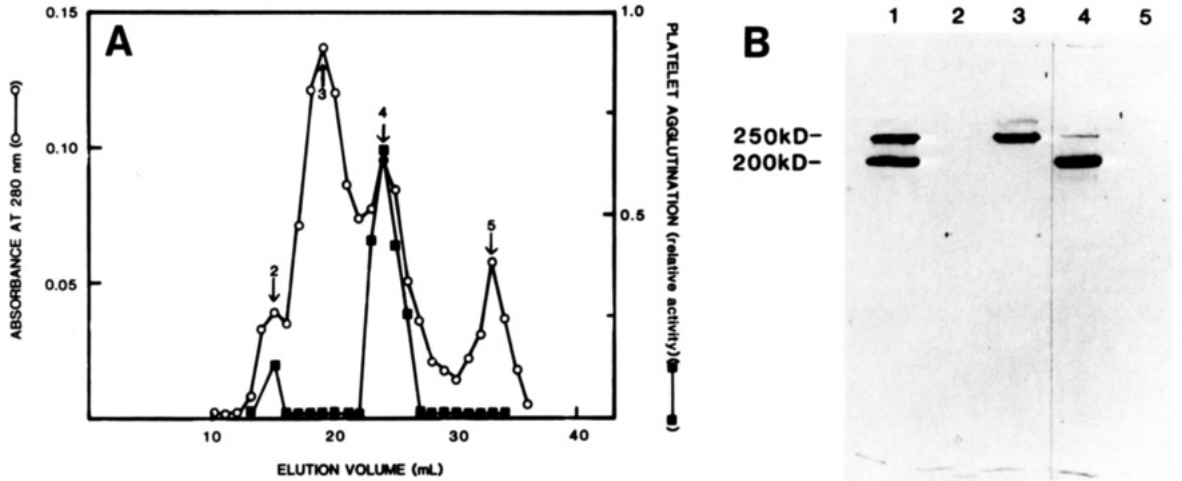

FIGURE 5: Analysis of P-I digest fragments for platelet agglutinating ability. (A) vWF (1.30 mg) was incubated for 24 h with P-I. Digestion was arrested by addition of EDTA to a final concentration of 2 mM. The digest was applied to a 0.9 × 47 cm Sepharose CL-6B column equilibrated in PBS. Fractions were assayed for platelet agglutinating activity. (B) Peak fractions (indicated by arrows) were electrophoresed on a 7.5% NaDodSO$_4$–polyacrylamide gel. The gel was stained with Coomassie Blue. Lane 1 is a precolumn sample.

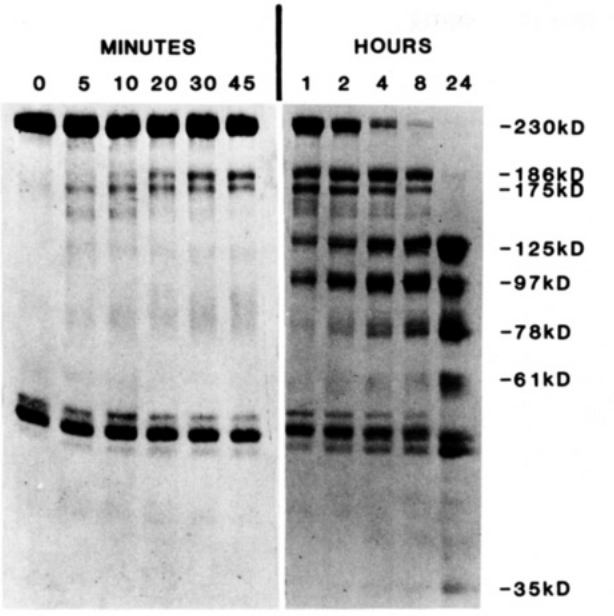

FIGURE 6: Polypeptide composition of a P-I digest of vWF. vWF (0.45 mg/mL) was incubated with P-I. At the indicated times, aliquots were removed and diluted with an equal volume of $NaDodSO_4$ diluent buffer containing 4 mM EDTA and 1% DTT. Samples were electrophoresed on a 7.5% $NaDodSO_4$–polyacrylamide gel. The gel was stained with Coomassie Blue.

gestion patterns of several preparations of vWF containing various amounts of the contaminant have been analyzed. The presence of the contaminant did not affect either the pattern or the quantity of cleavage products generated by P-I.

The intact vWF subunit (lane 1) had a molecular weight of 230K. P-I has four different cleavage sites in the vWF subunits. At the earliest time points (5–15 min), two intermediate cleavage products were generated, which had molecular weights of 175K and 186K, respectively. The 35- and 45-kDa polypeptides, which comprise part of the 200- and 250-kDa fragments, respectively, are probably generated from these initial cleavages, although they are not well visualized on the gels. The 35-kDa polypeptide was visible on this particular gel, but the 45-kDa polypeptide was not. Its presence was probably masked by the contaminant. The 186- and 175-kDa fragments were then cleaved, generating four polypeptides with molecular weights of 125K, 97K, 78K, and 61K. These four chains, along with the 35- and 45-kDa chains, are the six polypeptides which comprise the 250- and 200-kDa fragments.

Densitometric scanning of the lane containing the 24-h incubation sample showed that the stained intensity of the 125-, 97-, 78-, and 61-kDa polypeptide chains was proportional to their molecular weights. This indicated that they were present in approximately equimolar ratios. All four polypeptides stained strongly with periodate–Schiff, indicating the presence of carbohydrate. These four polypeptides were not detected at the early stages of digestion.

Analysis of Proteolytic Products of vWF Modified with ^{125}I-Labeled Bolton–Hunter Reagent. The fragmentation pattern of P-I-digested vWF was too complicated to discern which intermediate polypeptides the four terminal polypeptide chains were derived from. Their origin was deciphered by analysis of P-I digestion patterns of vWF labeled either with ^{125}I by the Iodogen method or with ^{125}I-labeled Bolton–Hunter reagent.

The source of the 125- and 97-kDa polypeptide chains was determined by studying the P-I digestion products of vWF modified with ^{125}I-labeled Bolton–Hunter reagent. Reduced samples of ^{125}I-labeled Bolton–Hunter-labeled vWF (^{125}I-BHR-vWF), which had been digested with P-I for various times, were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The autoradiogram of the gel is shown in Figure 7A. It revealed that regions of vWF which were modified by ^{125}I-BHR were present primarily in the 125- and 97-kDa chains of a terminal digest. Both the 186- and 175-kDa intermediate polypeptides contained label.

Each lane of the autoradiogram was scanned with a densitometer to determine the relative concentration of these polypeptides at each stage of the digestion (Figure 7B). The 125- and 97-kDa polypeptides contained 65% and 35%, respectively, of the total label, which on a molar basis is approximately a 1:1 stoichiometric ratio. The graph also revealed that the rates of formation and cleavage of the 175-kDa intermediate polypeptide were faster than those of the 186-kDa polypeptide. The rate of formation of the 97-kDa polypeptides correlated with the cleavage of the 175-kDa polypeptide, and the appearance of the 125-kDa chain correlated with the depletion of the 186-kDa polypeptide.

Analysis of the Proteolytic Products of ^{125}I-vWF. The origin of the 75-kDa polypeptide was determined by electro-

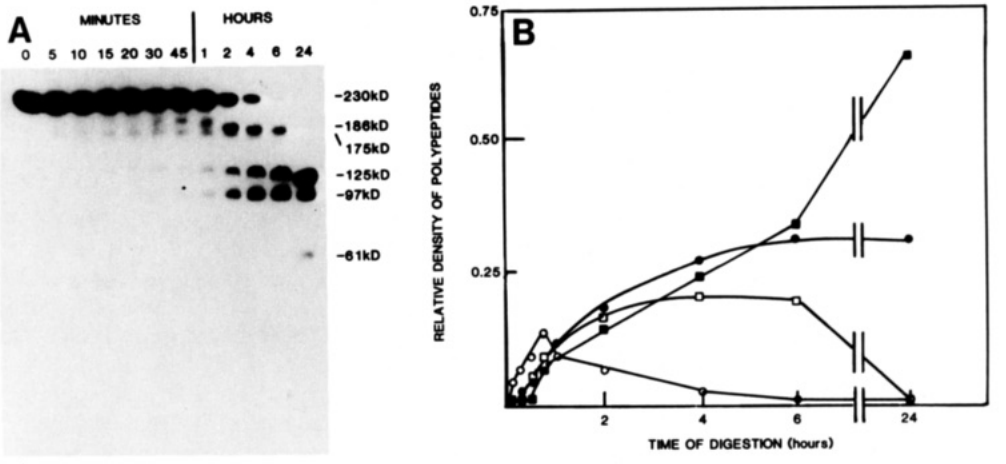

FIGURE 7: Time course of P-I digestion of ^{125}I-BHR-vWF. (A) A sample of vWF (512 μg), which had been labeled with ^{125}I-labeled Bolton–Hunter reagent (0.82 residue modified per subunit), was digested with P-I. At the times indicated, aliquots were removed and diluted with an equal volume of $NaDodSO_4$ diluent buffer containing 1% DTT and electrophoresed on a 7.5% $NaDodSO_4$–polyacrylamide gel. The gels were dried and bands visualized by autoradiography. (b) Each lane of the autoradiogram was scanned by a densitometer to determine the relative concentration of each fragment at each time point. The total density in each lane was normalized to the density of the intact subunit at zero time (lane 1), which was given a relative value of 1.0. (○) 175-kDa polypeptide; (●) 97-kDa polypeptide; (□) 186-kDa polypeptide; (■) 125-kDa polypeptide.

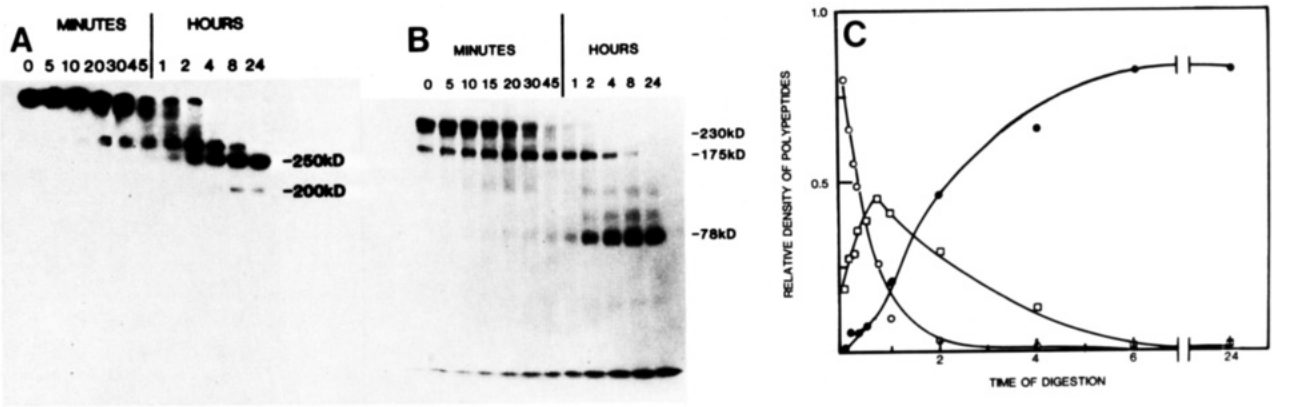

FIGURE 8: Time course of P-I digestion of ^{125}I-vWF. (A and B) P-I digest of ^{125}I-vWF. vWF (0.78 mg), containing ^{125}I-vWF (1.8×10^6 cpm), was incubated with P-I. At the times indicated, aliquots were removed and diluted with an equal volume of $NaDodSO_4$ diluent buffer without (A) or with (B) 1% DTT. The samples were electrophoresed on 7.5% $NaDodSO_4$–polyacrylamide gels. The gels were dried, and autoradiography was performed. (C) The autoradiogram (B) was scanned on the densitometer to determine the relative concentrations of the fragments at each time point. The density in each lane was calibrated relative to the density in lane 1. (○) 230 kDa; (□) 175 kDa; (●) 78 kDa.

phoretic analysis of vWF labeled with ^{125}I by the Iodogen procedure and digested with P-I for various times. The autoradiograms are shown in Figure 8A,B. The autoradiogram of the gel containing the nonreduced samples showed that the regions of the vWF subunit which were labeled were primarily contained within the 250-kDa fragment. The autoradiogram of the gel containing the reduced samples showed that the nondigested vWF used (zero time point) was partially proteolyzed since the preparation contained the 175-kDa polypeptide chain and a higher molecular weight fragment whose mobility was slightly faster than the intact subunit. These degradation products (and the 186-kDa polypeptide chain) are usually present in trace amounts in vWF preparations and are probably generated during purification. The autoradiogram revealed that the region or regions of the vWF subunit containing iodinated tyrosines were present primarily in the 175-kDa intermediate chain and the 78-kDa polypeptide chain. Densitometric scanning of the autoradiogram (Figure 8C) indicated that the formation of the 78-kDa polypeptide correlated with the degradation of the 175-kDa intermediate polypeptide chain. This experiment revealed that the 175-kDa intermediate and 78-kDa terminal polypeptides contain a region of vWF that is particularly susceptible to iodination. No other polypeptides which comprise the digest contained an amount of iodine equivalent to the 78-kDa polypeptide chain. The 186-kDa intermediate polypeptide chain and its cleavage products were visible by Coomassie staining, but they did not contain an amount of iodine equivalent to the 78-kDa polypeptide chain. The possibility existed that other small, labeled peptides were generated during digestion, but they diffused from the gel during the fixation procedure. To determine the existence of these peptides, an aliquot of a 24-h digest of ^{125}I-vWF was electrophoresed on an $NaDodSO_4$–polyacrylamide gel after reduction. The gel was sliced immediately after electrophoresis, and the slices were counted for γ emission. The results indicated that the majority of iodine (approximately 70%) was present in the 78-kDa polypeptide. No small peptides with specific activity equivalent to the 78-kDa polypeptide chain were detected. Molecular exclusion chromatography of digests of ^{125}I-vWF also did not reveal the presence of any small labeled fragments (data not shown).

Immunological Characterization of the Terminal P-I Digest Fragments. Immunological studies were performed with nine different monoclonal antibodies to elucidate whether their epitopes were present on either the 250-kDa or the 200-kDa fragment. Purified vWF and 250-, and 200-kDa fragments were spotted directly onto nitrocellulose paper, and the papers were incubated first with one of the monoclonal antibodies and then with ^{125}I-labeled goat anti-mouse IgG. As a control, the proteins were incubated with a monoclonal antibody directed against human factor XII. This antibody recognized neither native protein nor the fragments.

Table I: Specific Antibody Recognition of Fragments[a]

monoclonal antibody	intact vWF	250-kDa fragment	200-kDa fragment
1	16000	15000	1500
6	17000	16000	1900
7	12000	8000	600
9	7000	4000	100
10	13000	10000	8000
16	9000	200	7000

[a] Samples of vWF, 250-kDa fragment, and 200-kDa fragment were applied to nitrocellulose filter paper, and their recognition by monoclonal antibodies was performed as described in Figure 9. Specific antibody recognition is reported as cpm of second antibody bound to spots of 590 ng of the proteins and is corrected for cpm bound to nitrocellulose paper coated with BSA.

Monoclonal antibodies 2 and H-9 recognized both native vWF and the 200-kDa fragment, but not the 250-kDa fragment (Figure 9). Both monoclonal antibodies are directed against the region of vWF which binds to GPIb. The binding and agglutination studies discussed previously revealed that the 200-kDa fragment contained the region of vWF which binds to GPIb.

Monoclonal antibodies 1, 6, 7, 9, and 10 recognized the 250-kDa fragment, and monoclonal antibodies 10 and 16 recognized the 200-kDa fragment (Table I). These studies also revealed that monoclonal antibodies 1, 6, and 7 bound to the 250-kD fragment and native vWF with equal affinity. However, antibodies 9, 10, and 16 had a greater affinity for native vWF than the fragments, suggesting that the structure of these epitopes had been altered by proteolysis. Only one antibody (10) recognized both fragments.

Monoclonal antibody 7, whose epitope was located on the 250-kDa fragment, recognizes its epitope on both native and reduced, denatured vWF (Mascelli et al., 1986). Immunoblotting studies were performed with reduced samples of a time course of P-I digestion of vWF to elucidate which polypeptide chains contain the epitope for monoclonal antibody 7 (Figure 10). The autoradiogram revealed that the antibody recognized the 230-kDa subunit and both the 186- and 175-kDa intermediate polypeptides but only one terminal polypeptide, at 78

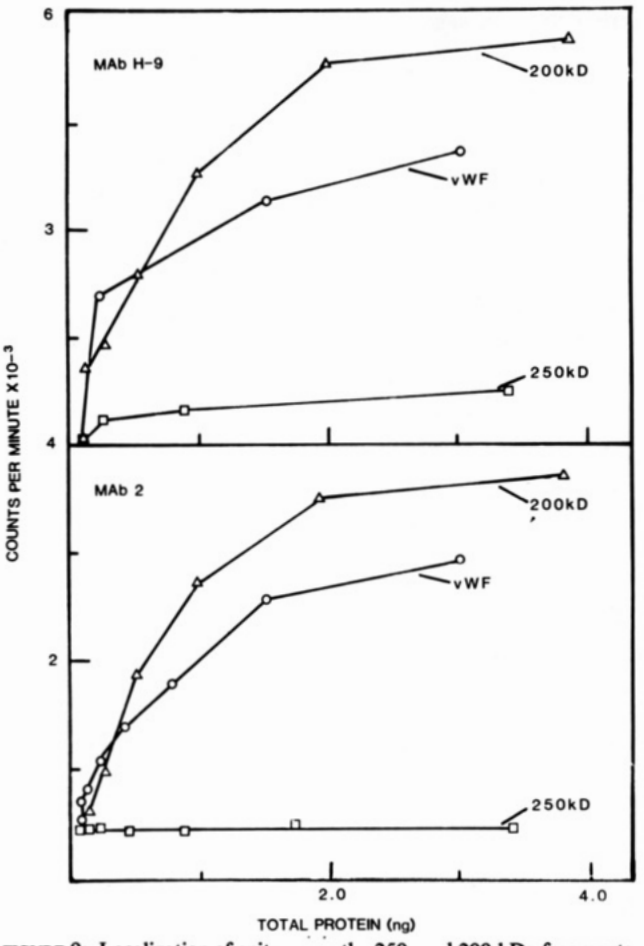

FIGURE 9: Localization of epitopes on the 250- and 200-kDa fragments. Serial dilutions of vWF (○), 250-kDa fragment (□), and 200-kDa fragment (△) (20 μL) were spotted onto nitrocellulose paper. Nonreacted sites on the nitrocellulose paper were blocked with PBS containing 3% BSA, and the strips were incubated individually with either monoclonal antibody 2 or H-9 (1 μg/mL), in immunoblotting buffer. The papers were then incubated with ^{125}I-labeled goat anti-mouse IgG antibody (1 × 10^6 cpm) in 100 mL of immunoblotting buffer, washed, dried, and autoradiographed to visualize the spots. Spots were cut out to determine the amount of second antibody bound.

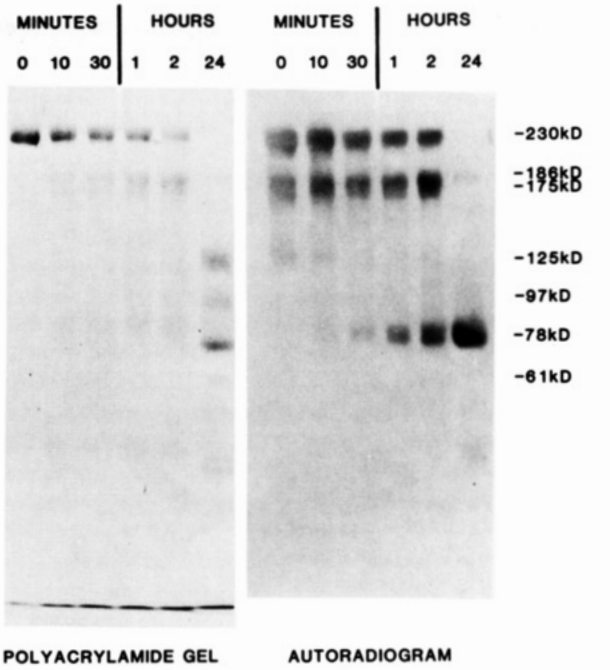

FIGURE 10: Localization of the epitope for monoclonal antibody 7 on the polypeptide chains of a P-I digest. vWF was incubated with P-I. At the times indicated, aliquots were removed and diluted with an equal volume of $NaDodSO_4$ diluent buffer containing 4 mM EDTA and 1% DTT. Duplicate samples were electrophoresed on a 7.5% $NaDodSO_4$–polyacrylamide gel. The gel was cut in two. The samples were either stained with Coomassie Blue or transferred to nitrocellulose filter paper. The paper was first incubated with monoclonal antibody 7 (1 μg/mL) and then with ^{125}I-labeled goat anti-mouse IgG (1 × 10^6 cpm) in 100 mL of immunoblotting buffer. The paper was then washed, dried, and autoradiographed.

kDa, which was derived from cleavage of the 175-kDa intermediate chain. These results suggest that all vWF subunits are recognized by monoclonal antibody 7. However, they also indicate that the 186- and 175-kDa intermediate polypeptides are cleaved at different sites. When the 175-kDa chain was cleaved, the area containing the epitope was present on the 78-kDa polypeptide chain. However, when the 186-kDa chain was cleaved, the monoclonal antibody 7 epitope was apparently destroyed, because none of the other terminal cleavage products was recognized by the antibody.

Discussion

Protease I is one of four proteases which were isolated from the venom of the Western Diamondback rattlesnake *Crotalus atrox* by Pandya and Budzynski (1984). The peptide specificity of this enzyme has not been determined, but Pandya showed that the protease makes only one cleavage in human fibrinogen. It is possible that protease I is the same enzyme as α-protease (M_r 23K), which was isolated from *C. atrox* venom by Pfleiderer and Sumyk (1961). This protease cleaves amide bonds of leucine, isoleucine, and valine.

From our data, it was possible to construct a model for the structure of bovine vWF. The model is shown in Figure 11. It identifies the fragment which contains the heparin and GPIb binding domains and the polypeptide chains which contain the regions of vWF modified by iodination or Bolton–Hunter reagent. It also shows the relative positions of the cleavage sites of P-I, and the epitopes for some of the monoclonal antibodies.

P-I makes two types of cleavages in each subunit. One type of cleavage occurs close to the end of the polypeptide chain and is within a disulfide-bridged region. The second cleavage is located toward the middle of the subunit and splits the molecule into two major fragments of M_r 250K and 200K.

The origins of three of the four largest polypeptide chains were revealed by the digestion patterns of the modified vWF. These results suggest that all vWF subunits are not cleaved by P-I in the same fashion. Half the subunits (type A) are first cleaved, generating the 35-kDa terminal polypeptide and the 186-kDa intermediate polypeptide, which when cleaved produce the 125- and 61-kDa terminal polypeptide chains. The other subunits (type B) are first cleaved to generate the 45-kDa terminal and 175-kDa intermediate polypeptide chains, which when cleaved produce the 97- and 78-kDa terminal polypeptide chains.

The P-I 250- and 200-kDa fragments are each composed of three disulfide-linked polypeptide chains. The 250-kD fragment is composed of a 45- and a 78-kDa polypeptide chain from one subunit and a 125-kDa polypeptide chain from an adjacent subunit, linked by disulfide bonds. The 200-kDa fragment is composed of a 35- and a 61-kDa polypeptide chain from one subunit, disulfide-linked to a 97-kDa polypeptide from an adjacent subunit.

The GPIb and heparin binding domains are present on only the 200-kDa fragment. Immunological studies also suggest that the two fragments, for the most part, are immunologically distinct. This evidence implies that the two fragments, like

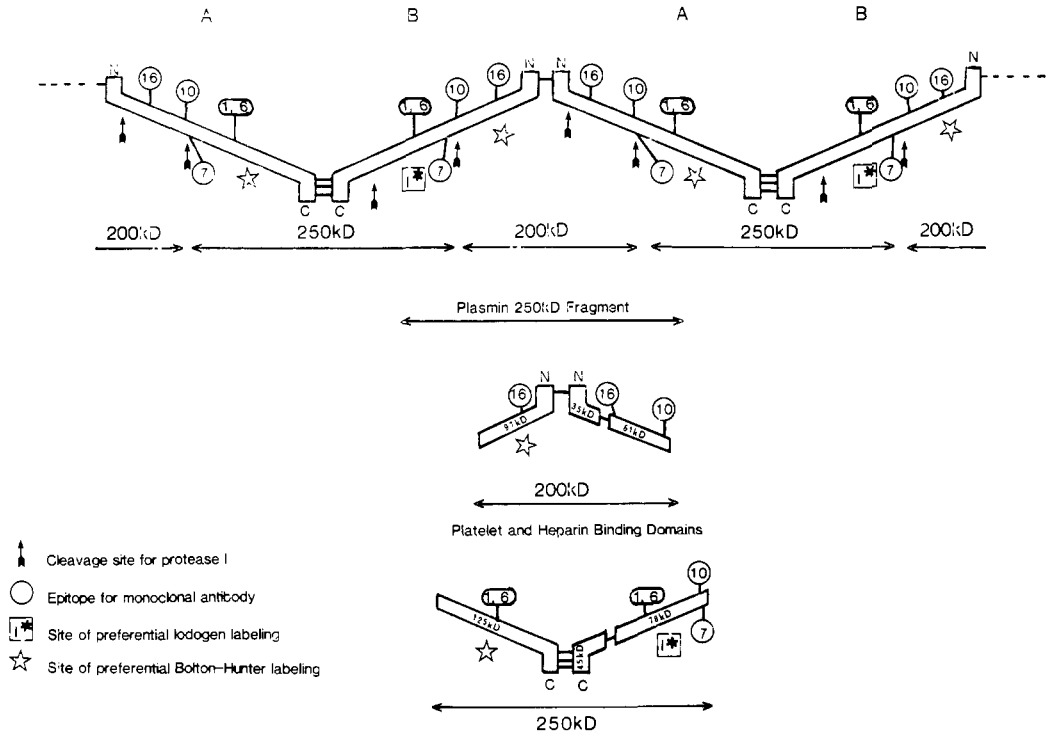

FIGURE 11: Diagram of a bovine vWF oligomer, illustrating P-I cleavage sites, cleavage products, polypeptides which are modified by iodination and Bolton–Hunter reagent, approximate location of GPIb and heparin binding domains, and approximate location of epitopes for monoclonal antibodies 1, 6, 7, 10, and 16.

the two V-8 protease fragments of human vWF, are composed of different regions of the vWF subunit. Since both bovine fragments are approximately the size of a subunit, they are probably homodimers, each consisting of either the amino- or the carboxyl-terminal region of two subunits. From these data, the model of bovine vWF is drawn in which the subunits are arranged in a head-to-head/tail-to-tail manner.

The immunological data suggest that adjacent subunits are cleaved differently. With the exception of one antibody, antibodies which recognize one fragment do not recognize the other, suggesting that the two major fragments are, for the most part, immunologically distinct. Competitive binding of the monoclonal antibodies of intact vWF revealed that monoclonal antibodies 1 and 6, whose epitopes are present on the 250-kDa fragment, partially compete with monoclonal antibody 10 for binding to vWF, suggesting that their epitopes may lie in close proximity (Mascelli et al., 1986). Monoclonal antibody 10 partially competes with antibody 16, whose epitope is present on the 200-kDa fragment. These results suggest that the epitope for monoclonal antibody 10 lies between antibodies 1 and 6 and antibody 16. Since monoclonal antibody 10 recognizes both fragments, the fragments probably share a similar region of the vWF subunit, in which part or all of this region comprises the epitope for monoclonal antibody 10. This overlapping sequence, whose size has not been determined, supports the existence of alternate cleavage sites on adjacent subunits. Identification of the polypeptides which contain the epitope for monoclonal antibody 7 indicates the intermediate polypeptides (M_r 186K and 175K) are cleaved at different sites.

This asymmetric cleavage pattern strongly implies that structural differences exist between adjacent subunits. Other evidence which supports this conclusion is the observation that the two sets of subunits are iodinated in a different manner. Only certain subunits (type B) contain a region which is particularly susceptible to iodination using Iodogen. This area is present on the 78-kDa polypeptide chain which comprises part of the P-I 250-kDa fragment.

Asymmetric cleavage patterns have also been observed with human vWF. Bockenstedt et al. (1986) found that the V-8 fragments of human vWF are not composed of two identical disulfide-linked chains. Their Sp II fragment (M_r 235K) is composed of three polypeptide chains (M_r 45.5K, 60K, and 110K), and their Sp III fragment (M_r 315K) is composed of two different chains (M_r 175K and 115K), suggesting that two other V-8 cleavage sites exist. Girma also reports the presence of these alternate cleavage products but concludes that they are only minor proteolytic products, since their concentration varies between different digest preparations. A possible explanation for this discrepancy is that their V-8 protease digests are not terminal but limited digests. That is, the protein is incubated for a limited period of time, so only preferential sites are cleaved. Therefore, the concentration of the extra cleavage products could vary, depending on the length of digestion.

The reason for the asymmetric cleavages in bovine vWF has not been determined. It could be due to a difference in the primary structure of the molecule. Among all cDNA clones of human vWF sequenced by investigators, there are eight nucleotide discrepancies which have been found. Of these, four result in single amino acid substitutions, of which three are located in the amino-terminal region and one in the carboxyl-terminal region of the polypeptide chain (Titani et al., 1986; Shelton-Inloes et al., 1986). However, none of these potential protein sequence polymorphisms either generates or destroys a potential cleavage site for protease I. Also, at present, there is no way to exclude the possibility that the discrepancies arose through errors of transcription during cDNA library preparations or that they represent nonfunctional mRNA sequences.

The asymmetric cleavage pattern could also be a result of differences in the tertiary structure of subunits. The protease makes only two cleavages in each subunit, indicating that it does possess a preference for cleavage of substrates. The tertiary structure of the area around the target amino acid is probably an important factor in this preference. The amino-

and carboxyl-terminal regions of subunits are very rich in cysteine residues, all of which are incorporated into intra- and interchain disulfide bridges.

According to Girma's results, the V-8 protease fragment (Sp 111, M_r 315K) of human vWF, which contains both heparin and GPIb binding domains, is composed of two identical 170-kDa polypeptide chains from the amino-terminal region of the subunit. Our 200-kDa fragment probably represents the amino-terminal portion of the bovine subunit. The tryptic peptide of human vWF isolated by Fujimura et al. (1986), which contains both the platelet GPIb and heparin binding domains, is located between amino acid residues 449 and 728. This same region of bovine vWF is located within the 200-kDa fragment.

The GPIIb/IIIa binding domain is present on Girma's smaller fragment (M_r 235K). This fragment is also a homodimer, composed of two identical polypeptide chains of 110 kDa from the carboxyl-terminal region of the subunit, and contains the tetrapeptide Arg-Gly-Asp-Ser which is involved in binding to GPIIb/IIIa. It is likely that our P-I 250-kDa fragment contains the carboxyl-terminal regions of the vWF subunits and that the tetrapeptide is contained within this fragment.

The P-I 250-kDa fragment elutes from a molecular exclusion column under nondenaturing conditions at a position equivalent to that of a protein whose molecular weight is 1×10^6, suggesting either that it is very asymmetric or that it associates noncovalently. The major fragment of bovine vWF which is generated by proteolysis with plasmin (Mascelli et al., 1986) also has a Stokes radius equivalent to a molecular weight of 1×10^6. We showed that at physiological ionic strength, the plasmic fragment associates into higher molecular weight oligomers, primarily tetramers. The tetramers can be dissociated by high ionic strength, suggesting that forces holding the plasmic fragments together are electrostatic. The data indicate that a region is present on the polypeptide chain which is responsible for noncovalent association of the vWF subunits. This association may play a role in the assembly of oligomers in vivo.

The platelet GPIb binding domain is present on the 200-kDa fragment. This fragment inhibited binding of native vWF to formalin-fixed platelets, but BSA or the 250-kDa fragment could not. The amount of 200-kDa fragments required to inhibit binding of ^{125}I-vWF by 50% (12.5 μg/mL, 54 nM) was 5 times greater than the amount of native vWF needed (2.2 μg/mL, 13 nM), indicating that its affinity for GPIb was 5 times less than native vWF. The decrease in affinity of the fragment could be due to proteolytic alteration of the binding site or the need for multivalent binding of covalently linked vWF subunits for high-affinity binding.

The 200-kDa fragment could agglutinate platelets, but its agglutinating ability was only 5% that of native vWF. The fragment has a molecular weight of 200K under nondenaturing conditions, suggesting that the fragments do not noncovalently associate to form oligomers. Since the fragment can agglutinate platelets, each 200-kDa fragment probably contains two GPIb binding domains. The decrease in agglutinating ability could be due to a decrease in affinity. Another possible explanation is that the large size of native vWF is more effective for spanning the distance between platelets imposed by electrostatic repulsion (Coller, 1983).

Monoclonal antibodies 2 and H-9, which inhibit platelet agglutination by bovine vWF, recognize the 200-kDa fragment, but not the 250-kDa fragment. Antibodies 2 and H-9 do not compete with one another for binding to vWF (Mascelli et al., 1986), indicating that binding of vWF to GPIb may require the functional participation of two different molecular loci on the vWF molecule. Similar distinctions have been made by Bowie et al. (1983), who analyzed several monoclonal antibodies to porcine vWF for inhibition of ristocetin cofactor activity and their effects on in vitro bleeding times.

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Registry No. vWF, 109319-16-6; heparin, 9005-49-6.

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