Structural Studies on the Functional Heterogeneity of von Willebrand Protein Polymers

By S. Eric Martin, Victor J. Marder, Charles W. Francis, and Grant H. Barlow

VON WILLEBRAND protein is a glycoprotein composed of subunits linked by disulfide bonds that may circulate in vivo as a series of polymers of molecular weight greater than 10^6. Larger molecular weight forms generally have greater in vitro potential for platelet interaction as reflected by increased ristocetin cofactor activity and greater affinity for binding to subendothelium. In vivo functional reflections of molecules of different size are indicated by the failure of factor VIII concentrates, which are relatively deficient in larger forms, to correct the bleeding time of patients with von Willebrand's disease, by variant IIA von Willebrand's disease in which the smaller molecular weight polymers predominate, and by the preferential removal of large molecular weight forms after treatment of a patient with acquired von Willebrand's disease, presumably as the result of their increased binding efficiency. Although some studies have noted the importance of disulfide bonds and of terminal sialic acid residues and penultimate galactose residues as important factors in the normal function of von Willebrand protein, the molecular basis for the distinction between high and low activity in large and small polymers has not been determined, nor have there been studies of individually separated polymers within these two broad functional or size categories. In the present report, individual polymers or similar groups of polymers purified from human cryoprecipitate are compared with regard to a number of possible contributory factors that could explain the observed differences in activity. Studies performed include evaluations of minor disulfide bound subunit polypeptide chains, active molecular fragments after tryptic degradation, content and susceptibility of carbohydrate, and disulfide bond contributions to overall activity. The data suggest that each polymer has its own level of ristocetin cofactor activity and that, in addition to overall size, tertiary structure as dictated by disulfide bond arrangement is an important determinant of differences in activity.

MATERIALS AND METHODS

Purification of Human von Willebrand Protein

Cryoprecipitate was prepared by the American Red Cross, Rochester Regional Blood Program and after washing with ethanol and absorption with aluminum hydroxide, it was processed by polyethylene glycol (PEG) 6000 (Carbowax, Fisher Scientific, King of Prussia, Penn.) precipitation and Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, N.J.) chromatography in β-alanine buffer (0.3 M β-alanine, 0.01 M sodium phosphate, 0.15 M NaCl, 0.02 M EACA, 10 U aprotinin/ml, 0.05% sodium azide, pH 6.8) as described previously to obtain von Willebrand protein. Protein determinations were performed according to the technique of Lowry. Factor VIII procoagulant activity was determined by the

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Supported in part by Grant #5-R01-HL21379-02 from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md.

Submitted July 11, 1980; accepted October 3, 1980.

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0006-4971/81/5702-0017$02.00/0
two-stage thromboplastin generation test and the ristocetin cofactor assay utilized formalinized platelets.

Antisera prepared in rabbits against human von Willebrand protein and fibrogen were adsorbed by passage through columns of Sepharose-CL4B beads (Pharmacia Fine Chemicals) coupled to per electrophoresis assuming 1 U of antigen and 0% ristocetin cofactor activity) were prepared as described before. Antisera against fibrogen and against cryoprecipitate from a patient with severe von Willebrand’s disease (absent von Willebrand antigen, 0% procoagulant activity, and 0% ristocetin cofactor activity) were prepared as described before. Antisera against human IgM, α2-macroglobulin, and β2-lipoprotein were obtained from Cappel Laboratories (Cochranville, Penn.). Von Willebrand antigen was measured by rocket immunodiffusion was performed as described by Ouchterlony and crossed immunoelectrophoresis according to previously described methods. The subunit composition of von Willebrand polymers was studied following the removal of immunoprecipitates from immunodiffusion plates, washing in 8.5 x 2.5 cm perforated plastic centrifuge tubes for 2 days with 1.0 M sodium chloride, 0.05 M tris buffer pH 7.2, then for 2 days with 0.004 M borate, 0.064 M tris buffer pH 8.6. The immunoprecipitates were then mixed with 0.05 mL of 6% sodium dodecyl sulfate (SDS, 1 recrystallized from ethanol) (Sigma Chemical Co., St. Louis, Mo.), 8.0 M urea (Fisher Scientific) and 7.3 M β-mercaptoethanol (β-ME) (type 1, Sigma Chemical Co.), heated at 100°C for 5 min and then at 60°C for 25 min before application to polycrylamide gradient gel electrophoretic systems. Areas of agarose that were free of visible immunoprecipitate were processed in parallel as controls.

Trypsin Hydrolysis

Purified von Willebrand protein was dialyzed overnight against 0.3 M β-alanine, 0.01 M phosphate, 0.15 M sodium chloride, 0.05% sodium azide pH 6.8 (β-alanine buffer), after which trypsin (bovine pancreas type 1, 10,000 N-benzoyl-L-arginine ethyl ester U/ml) (Sigma Chemical Co.) was added to a final concentration of 0.25 mg/mg von Willebrand protein and the mixture incubated at 37°C with constant stirring. Degradation was stopped with soybean trypsin inhibitor (STI) (type 1-S lot 77C-8000) (Sigma Chemical Co.) at a final concentration of 0.25 mg/ml.

Removal of Sialic Acid and Galactose Residues

Purified von Willebrand protein in β-alanine buffer pH 6.8, containing 0.02 M EACA and 10 U aprotinin/ml was treated with neuraminidase (type X, purified by affinity chromatography, 53 U/mg bovine submaxillary mucin activity) (Sigma Chemical Co.) at a final concentration of 11.5 U/mg protein and incubated at 37°C for 18 hr. After testing for ristocetin cofactor activity, the sample was exposed to galactose oxidase (LS00 04522, Worthington Biochemical Co., Freehold, N.J.) at a final concentration of 160 U/mg protein at 37°C for two additional hr and retested for ristocetin cofactor activity. As controls, samples were mixed with β-alanine buffer in the absence of enzymes and incubated for appropriate intervals in parallel with test materials. The neuraminidase and galactose oxidase preparations used in the presence of 10 U of aprotinin/ml and 0.02 M EACA showed no evidence of proteolytic activity, as demonstrated by lack of degradation of 14C-labeled hemoglobin.

Limited Disulfide Bond Reduction

Purified von Willebrand protein was incubated with 0.1 M β-ME at 37°C and 0.025-ml and 0.05-ml aliquots drawn at timed intervals.

The 0.025-ml aliquot was mixed with an equal volume of 0.05 M 2-iodoacetamide (2-IAA) (Eastman Kodak Co., Rochester, N.Y.), incubated at 25°C for 3 min, mixed with 0.025 ml of bovine serum albumin (20 mg/ml) (fraction V, Sigma Chemical Co.), and used for determination of ristocetin cofactor activity. The 0.05-ml aliquot was mixed with 0.005 ml of 1.0 M 2-IAA, incubated at 25°C for 3 min, then added to 0.005 ml of 10 M urea, 6% SDS, 0.04 M borate, 0.32 M tris buffer at pH 8.6 before application to nonreduced polyacrylamide-agarose (2%-0.5%) gel electrophoresis. In other experiments the von Willebrand protein was treated with DTT (5 x 10^-4 M final concentration) and incubated at 25°C. Aliquots were drawn at timed intervals, mixed with 2-IAA (10^-4 M final concentration), tested for ristocetin cofactor activity and run in nonreduced SDS polyacrylamide-agarose gel electrophoresis.

Sucrose Gradient Ultracentrifugation

Von Willebrand protein samples were layered on 5%-20% sucrose gradients prepared manually in β-alanine buffer, and run in the SW 27.1 rotor (Beckman Model LS-65 ultracentrifuge) at a constant w²t of 44,500 rad²/sec x 10^3. Following centrifugation 1.0 ml fractions were collected from the top using a density gradient fractionator (Model 185, Instrumentation Specialties, Lincoln, Neb.), tested for ristocetin cofactor activity and run in nonreduced SDS polyacrylamide-agarose (2%-0.5%) gel electrophoresis.

Polyacrylamide Gel Electrophoresis

Polyacrylamide-agarose (2%-0.5%) slab or tube gels were prepared as previously described. Test samples were dissolved in urea-SDS-tris-borate buffer (2 M urea, 0.6% SDS, 0.008 M borate, 0.13 M tris final concentrations) and electrophoresis conducted towards the anode in a continuous buffer system of 0.1% SDS, 0.004 M boric acid, 0.064 M tris, pH 8.6, using 150-V constant voltage and stopped when the tracking marker (bromphenol blue) reached the end of the gel.

Polyacrylamide gradient slabs with linear concentration gradients of 5%-15% were prepared as previously described. When a nonreduced system was used, samples were dissolved in 0.6% SDS, 2 M urea, 0.025% NaN₃-EDTA in 0.008 M boric acid, 0.13 M tris buffer (final concentrations) at pH 8.6. When a complete disulfide-bond reducing system was employed, dithiothreitol (DTT) (final concentr...
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Densitometric Analysis of Electrophoretic Bands

Gel strips containing the Coomassie blue and PAS stained bands were scanned at 575 nm and 542 nm, respectively, in a Beckman Model 24 spectrophotometer equipped with a linear scanning device and recorder. Following scanning, the area under each peak was calculated with a planimeter (Model 39231, Coy Laboratory Products, Ann Arbor, Mich.), and the result used as a reflection of protein concentration in the gel strip.

RESULTS

Material containing factor VIII procoagulant activity, ristocetin cofactor activity, and von Willebrand antigen emerged from Sepharose CL-2B columns at 1.4 void volumes, after which it was collected into nine pools (Fig. 1) and concentrated by dialysis against 20% PEG-20,000. Double diffusion or immunoelectrophoresis in agar gels against appropriately adsorbed antisera showed that pools I-VII contained less than 1% IgM, fibrinogen, a2-macroglobulin, fibronectin, and β-lipoprotein, and did not react with rabbit antiserum against cryoprecipitate obtained from a patient.
with severe type I von Willebrand's disease who has a bleeding time greater than 15 min, 0% factor VIII procoagulant activity, 0% ristocetin cofactor activity, and 0% von Willebrand antigen by Laurell assay. Pool VIII reacted with anti-IgM and anti-fibrinogen antiserum, and pool IX with these and anti-fibronectin antiserum as well.

Analysis of the concentrated pools by SDS-electrophoresis in 2% polyacrylamide:0.5% agarose gels (Fig. 2, top) showed a series of bands of molecular size from $2.4 \times 10^6$ to greater than $10 \times 10^6$, based on the relative migration distances of IgM and the fibrin polymers as standards. Pools I-III contained the highest molecular weight forms, much of which was beyond the measurable limit of the standards and which was estimated as greater than $10 \times 10^6$. Pools IV-VI were composed mostly of intermediate sized polymers of minimum size $4.6 \times 10^5$, and pools VII-IX contained the smallest species of molecular size below $4.6 \times 10^5$. Small amounts of IgM (950,000), fibronectin (440,000), fibrinogen (340,000), and fibrin dimer (680,000) were present in pools VIII and IX. Densitometric analysis showed 2% and 14.8% IgM, 1 and 5% fibrinogen species, and 0 and 0.2% fibronectin in pools VIII and IX, respectively.

The disulfide bond-reduced subunits of the different groups of von Willebrand polymers was studied using a discontinuous SDS-polyacrylamide gradient gel system (Fig. 2, bottom). In addition to the major subunit of molecular weight 208,000, bands of 197,000, 174,000, and 154,000 were present in all pools, and a faint band of 130,000 was observed in all but pool I. The minor reduced bands were demonstrated least well in pool I because the protein concentration was only 20% that of the other pools. A band of 90,000 and bands corresponding to the IgM heavy chain and to reduced fibrinogen chains were clearly present in pool IX, faint in pool VIII.

The bands of 197,000, 174,000, and 154,000 were recovered along with that of 208,000 from reduced immunoprecipitates obtained after reaction with monospecific anti-von Willebrand antiserum (Fig. 3), suggesting that they represent von Willebrand protein. Bands that were faintly demonstrated in Fig. 2 were not visible in these immunoprecipitates.

The electrophoretic mobility of von Willebrand protein present in the separated pools corresponded to that of von Willebrand protein present in normal plasma (Fig. 4). Pool I showed more heterogeneity than was suggested by SDS-gel electrophoresis (Fig. 2, top), and the material in pools I and IX appeared to migrate as two populations, corresponding to slow and fast portions of the normal plasma pattern. Pool I had a predominance of the slow peak and pool IX showed primarily the fast peak.

The absolute ristocetin cofactor activity, total protein, Laurell von Willebrand antigen reaction, and densitometric quantity of 208,000 subunit chain per unit volume of each pool after concentration dialysis are shown in Table I. Specific ristocetin cofactor activities relative to each parameter of von Willebrand protein concentration are shown in Figs. 5A and B. Ristocetin cofactor activity was greatest in pool I by
Table 1. Ristocetin Cofactor Activity and Three Independent Estimates of von Willebrand Protein Concentration in Pooled Fractions Obtained by Sepharose CL-2B Elution (Fig. 1)

<table>
<thead>
<tr>
<th>Pool</th>
<th>Ristocetin Cofactor Activity (U/ml)</th>
<th>Lowry vW antigen (mg/ml)</th>
<th>vW antigen (U/ml)</th>
<th>208,000 Reduced Chain (Area U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>0.024</td>
<td>1.6</td>
<td>0.0554</td>
</tr>
<tr>
<td>II</td>
<td>47</td>
<td>0.360</td>
<td>17.8</td>
<td>0.7472</td>
</tr>
<tr>
<td>III</td>
<td>56</td>
<td>0.390</td>
<td>33</td>
<td>1.1038</td>
</tr>
<tr>
<td>IV</td>
<td>59</td>
<td>0.490</td>
<td>57</td>
<td>2.1890</td>
</tr>
<tr>
<td>V</td>
<td>57</td>
<td>0.530</td>
<td>50</td>
<td>1.8888</td>
</tr>
<tr>
<td>VI</td>
<td>59</td>
<td>0.540</td>
<td>42</td>
<td>1.5099</td>
</tr>
<tr>
<td>VII</td>
<td>57</td>
<td>0.430</td>
<td>63</td>
<td>2.3058</td>
</tr>
<tr>
<td>VIII</td>
<td>48</td>
<td>0.610</td>
<td>52</td>
<td>1.4933</td>
</tr>
<tr>
<td>IX</td>
<td>23</td>
<td>0.650</td>
<td>39</td>
<td>1.2147</td>
</tr>
</tbody>
</table>

VIII and IX lower. To rule out the possibility of inhibition of activity by fibrinogen, fibronectin, or IgM in pools VIII or IX, the samples were also tested using severe von Willebrand's disease plasma as the diluent; the results were identical as with a buffered diluent (Fig. 5A, dotted open circles).

A different specific activity for each polymer could not be proven or ruled out by analysis of the total pools, so the relative activity of individual polymers of von Willebrand protein was further evaluated by sucrose density gradient centrifugation of pools III and IX. Fractions that contained ristocetin cofactor activity were electrophoresed in nonreduced SDS 2% acrylamide: 0.5% agarose gels and the amount of von Willebrand protein was quantitated by densitometric analysis of the stained gel patterns (Fig. 6). Within the group of polymers present in pool IX (Fig. 2), the bands of 2.4, 3.4, and 4.6 \times 10^6 were associated with progressively greater specific activities, all of which were less than that associated with the 6.4 \times 10^6 band in pool III.

The absolute and relative concentrations of the minor reduced polypeptide chains of 197,000, 174,000, and 154,000 were analyzed by densitometric quantitation of each pool (Fig. 2, bottom). All pools had the same contribution of minor bands, approximately 1% of the total protein, and the same proportion of each of the three moieties relative to the 208,000 band. Therefore, the differences in ristocetin cofactor activity for pools of different polymer size could not be explained by functional enhancement or deficiency attributable to these minor polypeptide components.

The nonreduced SDS polyacrylamide gel pattern of 45-min tryptic hydrolysates of pools II, III, VIII, and IX showed the same degradation fragments of molecular weight 235,000, 219,000, 170,000 (faint), 154,000 (faint), 116,000, 43,000, and 22,000–26,000 (Fig. 7).
Fig. 5. Specific ristocetin cofactor activity of the pools of von Willebrand protein eluted from Sepharose CL-2B gels, as shown in Figs. 1 and 2. The left panel shows activity relative to the densitometric assay of reduced 208,000 subunit in each pool (solid circles) or to the amount of von Willebrand antigen detected by Laurell immunoelectrophoresis (open triangles). The dotted circles indicate results obtained for pools II, VIII, and IX, diluted in severe von Willebrand's disease plasma rather than in buffer, estimated on the basis of 208,000 subunit quantity. The right panel shows the ristocetin cofactor activity per mg of total protein in each pool, as determined by the Lowry technique.

Fig. 6. Nonreduced SDS polyacrylamide-agarose gels (2%-0.5%) of fractions obtained following simultaneous sucrose gradient ultracentrifugation of pools III and IX. Samples of 0.1 ml were diluted in urea-SDS-tris-borate buffer pH 8.6, applied to a disc gel, electrophoresed and measured densitometrically in comparison with the ristocetin cofactor activity of each fraction. The values at the bottom represent the activity of each fraction in comparison with the value obtained for sucrose gradient fraction 6.
FIG. 7. SDS-polyacrylamide gel electrophoresis of nonreduced von Willebrand protein pools II, III, VIII, and IX after degradation with trypsin (0.25 g/mg substrate) at 37°C for 45 min. Electrophoresis was performed on a discontinuous 5%-15% polyacrylamide gradient gel system using 12, 13, 20, and 22 μg of the respective digests. The residual ristocetin cofactor activity of each pool, expressed in absolute units per unit volume of sample and relative to the amount of 116,000 fragment in the digest as analyzed densitometrically are indicated at the bottom.

Residual ristocetin cofactor activity of each pool calculated in proportion to volume or to the active component of 116,000 molecular weight showed no significant difference between samples and no trend of increasing or decreasing activity in relation to size of the parent, untreated polymers.

The carbohydrate content of the different polymers was determined densitometrically by the ratio of PAS to Coomassie blue staining (Fig. 8). Despite differences in specific ristocetin cofactor activity, the ratio for each group or individual von Willebrand polymer was similar, most apparent in the analysis of pools VII and VIII. Fibronectin (CIG) had a ratio of 0.06, indicating a lower relatively content of carbohydrate than in von Willebrand protein. The ratio for IgM (0.12) and fibrinogen (0.13) were higher than for fibronectin, but still lower than the value obtained for von Willebrand polymers. The response of ristocetin cofactor activity to the removal of terminal sialic acid and penultimate galactose residues of polymers present in pools II and VIII was studied by sequential treatment with neuraminidase and galactose oxidase (Table 2). The loss of activity with galactose oxidase occurred only after prior liberation of sialic acid residues, and the effect of removing both of these residues on the ristocetin cofactor activity was similar for these groups of polymers, widely disparate in their molecular size. There was no alteration in electrophoretic mobility of von Willebrand protein using an SDS-polyacrylamide:agarose (2%-0.5%) system after incubation with either neuraminidase or galactose oxidase.

The relationship of disulfide bond structure to activ-
ity was evaluated by progressive exposure of polymers in pool II to either dithiothreitol or β-mercaptoethanol, followed by alkylation with 2-iodoacetamide. The ristocetin cofactor activity of the partially reduced pool II polymers was expressed relative to the activity of untreated pool IX polymers (Fig. 9). Before reduction, pool II had sixfold greater ristocetin cofactor activity than pool IX. After 2 min, the pool II polymers had decreased from an initial size of greater than $10 \times 10^6$ to a group with apparent molecular weight of $2.4-4 \times 10^6$. Although this partially-reduced pool II resembled in size the polymers of pool IX, they retained more than four-fold higher specific activity. After 5 min exposure to the reducing agent, most of the polymers in pool II were of molecular size $3.4 \times 10^6$, with lesser amounts of material of $400,000$ and $2.4 \times 10^6$. Pool IX had generally larger forms than was present in the 5-min pool II sample, consisting predominantly of bands of $2.4$ and $3.4 \times 10^6$ with contributions of $1.1$ and $4.6 \times 10^6$. Despite the smaller size of most forms present in partially reduced pool II, its ristocetin cofactor activity was still slightly higher, (1.4:1) than in pool IX. With the further decrease in the $2.4 \times 10^6$ moiety and the relative increase in that of $400,000$, the relative activity of reduced pool II continued to decrease. However, at 15 min the activity was still only slightly lower (0.7) than in pool IX, although the overall molecular size in pool II was lower than that in pool IX.

**DISCUSSION**

The factor VIII-von Willebrand protein that forms the basis of this report consisted of a series of multimeric structures that ranged in molecular weight from $2.4 \times 10^6$ to much greater than $10 \times 10^6$, as measured by comparative electrophoretic mobility of glutaraldehyde crosslinked IgM polymers (Fig. 2). This heterogeneous population of molecules obtained from human cryoprecipitate was analogous in size to those seen by

| Table 2: Effect of Neuraminidase and Galactose Oxidase on Ristocetin Cofactor Activity of Selected von Willebrand Polymers |
|-----------------------------------------------|---------------|---------------|
| Neuraminidase | Galactose Oxidase | Neuraminidase Followed by Galactose Oxidase |
| Pool II | 36 | 0 | 95 |
| Pool VIII | 33 | 0 | 93 |
other investigators\(^1\)\(^\text{--}^9\) and overlapped in electrophoretic position with the protein demonstrated in normal plasma by crossed-immunoelectrophoresis (Fig. 4). We did not distinguish a moiety of 10\(^6\) molecular weight in our preparation, suggesting that its concentration in cryoprecipitate is lower than in plasma. Its presence in the latest eluting pool prior to the major protein peak (Fig. 1) may have been masked by IgM (Fig. 2).

Electrophoretic analysis of the subunit composition of the different polymers using a polyacrylamide-agarose gel system (Fig. 2) showed a major subunit of molecular weight 208,000, but also other minor components, most evident of which were those of molecular weight 197,000, 174,000, and 154,000. The latter probably do not represent unrelated molecules that bind nonspecifically to von Willebrand protein, since they were present in immunoprecipitates of the purified material using monospecific anti-von Willebrand antibody (Fig. 3), and since they did not precipitate with antibody against plasma of severe von Willebrand's disease patients. In fact, they seemed to precipitate more efficiently with anti-von Willebrand antiserum than did the major subunit band. Minor components have been previously observed by other investigators\(^1\)\(^\text{--}^9\) and the tryptic peptide mapping studies of Gorman and Ekert\(^29\) suggested a structural relationship between the 208,000 subunit and the minor chains in the molecular weight range of 120,000–180,000. In our preparations, minor bands comprised only about 1% of the total subunit composition as measured by densitometric analysis in comparison with the 208,000 band, and probably do not represent in vitro proteolytic degradation, since they were consistently demonstrated despite the use of proteolytic inhibitors throughout the purification procedure. Furthermore, detailed studies of plasmic degradation in vitro\(^30\)\(^,\)\(^31\) show that the initial subunit chain is degraded through a series of smaller chains that do not correspond in size to the minor bands identified in Fig. 2, making it unlikely that they represent plasmic cleavage products, either in vivo or in vitro. Whether these bands are also present in von Willebrand protein freshly prepared from plasma has not been established.

Analysis of the ristocetin cofactor activity of the
polymers showed three possible levels of activity (Fig. 5), with those larger than $10 \times 10^6$ having the greatest activity and the smallest (less than $4.6 \times 10^6$) having the least activity. These differences in specific activity could be ascertained on the basis of von Willebrand antigen content or by densitometric measure of the 208,000 subunit, but they were not as apparent using a Lowry determination of protein concentration. Further separation of the polymers by sucrose gradient centrifugation (Fig. 6) was needed to establish that individual polymers probably have specific levels of ristocetin cofactor activity, with overlapping or similar activities of the pools reflecting the effect of groups of polymers (Fig. 5). These differences in the specific activity are not explained by the presence of contaminant proteins such as fibrinogen$^{33}$ in pools of the lower molecular weight polymers, since there was no difference in ristocetin cofactor activity using von Willebrand's disease plasma or buffer as the diluent (Fig. 5A). The findings of lower specific activity for smaller polymers are in concert with observations that show preferential binding of larger forms of von Willebrand protein with platelets$^{10,11}$ and in vivo removal of larger polymers after transfusion of cryoprecipitate into a patient with acquired von Willebrand's disease.$^{13}$

The experiments reported here were designed to investigate whether the differences in specific activity were due to a strict correlation between polymer size and function and/or whether other factors contributed to the activity, such as might be demonstrated by proteolytic degradation, partial disulfide bond reduction, or analysis of carbohydrate content. Heterogeneity in the subunit composition does not explain the observed differences, since the proportion of minor reduced bands of 197,000, 174,000, and 154,000 relative to the 208,000 band was constant for all samples. Thus, smaller polymers do not owe their decreased size or function to a greater degree of subunit variation.

All polymer groups of molecules contained the same proportion of carbohydrate, as reflected in PAS-staining of the gels after electrophoresis (Fig. 8), and they had similar sensitivity to the loss of sialic acid and the galactose residues (Table 2). These results are in agreement with observations by Zimmerman and colleagues who showed no significant difference in carbohydrate content of reduced von Willebrand protein obtained from normal individuals and from patients with variants of von Willebrand's disease.$^{33}$ Taken together, the data suggest that differences in carbohydrate content do not explain the functional difference between normal and variant molecules of patients, or between von Willebrand molecules of different molecular size from normal individuals.

Possible differences in primary structure of the various pools were also assessed by tryptic degradation, analyzed by gel electrophoresis and ristocetin cofactor activity determinations. Ristocetin cofactor activity is known to decrease rapidly upon exposure to trypsin,$^{18,30,34}$ the activity in early digests correlating with the presence of fragments of molecular weight greater than 314,000.$^{18}$ In the late stages of degradation, residual ristocetin cofactor activity accounts for approximately 5% of initial activity and correlates with the presence of a fragment of molecular weight 116,000.$^{18}$ Fig. 7 showed that the degradation products obtained after 45 min of exposure to trypsin were the same for all of the von Willebrand protein polymers. Residual ristocetin cofactor activity was accounted for by the 116,000 molecular weight fragment in each digest, and no difference was seen in the specific activity of this fragment regardless of the initial polymer size. This indicates that the difference in activity of the original undegraded polymers did not result from differences in the structure or specific activity of the 116,000 region of the molecules, and that enzyme-sensitive regions or exposed cleavage sites are similar for all forms, regardless of size.

There was a progressive loss in ristocetin cofactor activity with partial disulfide bond reduction and alkylation of the polymers, such as occurred with those of greater than $10 \times 10^6$ in pool II (Fig. 9). This is in agreement with Counts et al.$^6$ who noted the correlation of decreased activity with decreasing molecular weight during disulfide bond reduction. We have additionally found that the molecular size of such partially-reduced moieties is not the sole determinant of ristocetin cofactor activity, for instance, molecules obtained by reduction of pool II polymers were similar in size to molecules in unreduced pool IX, but they retained higher specific activity. Furthermore, when the pool II polymers were reduced to a general size of less than $1.1 \times 10^6$, clearly less than the mean size of polymers in pool IX, both samples had approximately the same ristocetin cofactor activity (Fig. 9). Thus, the disulfide bond organization of the polymers plays a major role in platelet-related activity. This would be consistent with the report of Cooper et al.$^{35}$ who noted an initial increase in activity of bovine von Willebrand protein after short exposure to mercaptoethanol, followed by the expected decrease in activity with continued disulfide bond reduction. This suggests that a more optimal conformation for platelet interaction may even exist with partial disulfide bond reduction than with the initial form of the molecule. Our results indicate that differences in the specific activity of the polymeric forms of von Willebrand protein found in cryoprecipitate are a function not only of their size but also of quaternary conformation that may be dictated by disulfide bond arrangements.
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Structural studies of the functional heterogeneity of von Willebrand protein polymers

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