Electron Microscopy of Human Factor VIII/Von Willebrand Glycoprotein: Effect of Reducing Reagents on Structure and Function

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ABSTRACT The structure of native and progressively reduced human factor VIII/von Willebrand factor (FVIII/vWF) was examined by electron microscopy and SDS gel electrophoresis and then correlated with its biological activities. Highly resolved electron micrographs of well-spaced, rotary-shadowed FVIII/vWF molecules showed their structure to consist of a very flexible filament that contains irregularly spaced small nodules. Filaments ranged from 50 to 1,150 nm with a mean length of 478 nm and lacked fixed, large globular domains as seen in fibrinogen and IgM. A population of multimeric FVIII/vWF species ranging in molecular weight from 1 to 5 million daltons and differing in size alternately by one and two subunits was observed on SDS-agarose gel electrophoresis. With progressive reduction of disulfide bonds by dithiothreitol (DTT), the electron microscopic size of FVIII/vWF decreased in parallel with increased electrophoretic mobility on SDS-agarose gels; between 0.1 and 0.5 mM DTT its structure changed from predominantly fibrillar species to large nodular forms. A 50% loss of vWF specific activity and FVIII procoagulant activity occurred at 0.4 mM DTT and 1 mM DTT, respectively, corresponding to the reduction of 4 and 12 disulfide bonds of the 62 disulfides per 200,000-dalton subunit. We conclude that reduction of a few critical disulfide bonds results in a major structural change by electron microscopy and a concomitant loss of ~50% of the vWF function.

Factor VIII/von Willebrand factor (FVIII/vWF) is a circulating complex glycoprotein that has two hemostatic functions: (a) it corrects the plasma defect in classic hemophilia; and (b) it shortens the prolonged bleeding time in patients with von Willebrand's disease (1). Specifically, FVIII procoagulant activity markedly accelerates the generation of activated factor X which then increases the rate of prothrombin cleavage to yield thrombin (2). The functional status of vWF can be assessed in vitro using the antibiotic ristocetin, which promotes the aggregation of platelets in the presence of normal vWF glycoprotein (3). In general, low levels of ristocetin-induced platelet aggregating activity correlate with the prolongation of the bleeding time in most patients with von Willebrand's disease (4). Purified FVIII/vWF exists as a family of multimers estimated to range in molecular weight from 1 to 20 million (5, 6). When fully reduced, however, the subunit molecular weight is 200,000 (7). The FVIII and vWF activities of the FVIII/vWF protein can be separated under a variety of unusual conditions; 99% of the protein possesses vWF activity while <1% gives rise to FVIII procoagulant activity (8). Many of the physicochemical properties of the vWF glycoprotein are known, but to date the FVIII procoagulant protein has not been purified and its biochemical properties remain undefined (9).

Studies from several laboratories have shown that the FVIII and vWF activities are differentially affected by certain modifications of the intact FVIII/vWF complex; in some instances, these efforts have allowed structure-function correlations to be made. For example, FVIII procoagulant activity is extremely sensitive to proteolytic degradation, but the ristocetin-induced platelet aggregating activity is relatively stable despite cleavage of the FVIII/vWF subunits to a much smaller size (10). In contrast, removal of carbohydrate decreases the platelet aggregating activity of the FVIII/vWF complex with no loss of FVIII procoagulant activity (11-13). Recently the effect on
FVIII/vWF structure and function by disulfide bond reduction has been examined (5, 14–21). There is general agreement that reduction with 1 mM dithiothreitol (DTT) results in a rapid loss of vWF activity and a decrease in the size of FVIII/vWF as measured by gel-filtration chromatography, sucrose density gradient ultracentrifugation, or agarose gel electrophoresis. There is no such consensus on the effect of reducing reagents on FVIII procoagulant activity; some suggest that disulfide bond reduction has little or no effect on this activity (5, 16, 17, 19, 20), but three laboratories indicate that FVIII procoagulant activity is abolished by DTT concentrations ranging from 1 to 50 mM (14, 15, 18).

There have been several attempts made to resolve the FVIII/vWF structure by electron microscopy (22–25), but none have employed the recently described improved rotary shadowing technique that gives a very reproducible spread of clear, well-separated molecules (26, 27). Now, besides presenting the first highly resolved electron micrographs of individual native FVIII/vWF molecules, we also report the effect of reducing reagents on the structure of FVIII/vWF and correlate these changes with those detected by sodium dodecyl sulfate (SDS) gel electrophoresis as well as with the progressive loss of both FVIII and vWF activities.

MATERIALS AND METHODS

Materials

Intermediate-purity human FVIII/vWF concentrates and platelet-rich plasma were obtained from the American Red Cross. Ristocetin was purchased from H. Lundbeck and Co. (Copenhagen, Denmark) and SeaKem agarose from MCI Biomedical (Rockland, MD). [3H]iodoacetic acid and carrier-free Na125I were the products of New England Nuclear (Boston, MA) and Amersham Corp. (Arlington Heights, IL), respectively. Biogel A15m (4% agarose) was purchased from Bio-Rad Laboratories (Richmond, CA). Sepharose-bound lactoperoxidase was obtained from Millipore Corporation (Bedford, MA). Human IgM was the generous gift of Dr. R. Buckley (Duke Medical Center). Fibrinogen was purchased from LKB Instruments, Inc. (Rockville, MD). 2-mercaptoethanol was purchased from Sigma Chemical Co. (St. Louis, MO) within 6 months of this study and stored at 4°C. All other chemicals were reagent grade or better and used without further purification.

Purification and Characterization of Human FVIII/vWF

Starting with intermediate-purity concentrates (28), FVIII/vWF was purified by polyethylene glycol precipitation and 4% agarose gel chromatography as previously described (29). Highly purified FVIII/vWF was consistently obtained and validated by SDS gel electrophoresis (29). FVIII/vWF protein concentrations were estimated from absorbance at 280 nm minus the absorbance at 320 nm using an extinction coefficient (E280) of 1.23; this method agrees well with values given by amino acid analysis or the Lowry protein assay (30). FVIII procoagulant activity was measured by the partial thromboplastin time method of Langdell et al. (31). Hemophilic plasma with <1% FVIII procoagulant activity was used as substrate and was activated before use by incubation for 6 min at 37°C with 5 mg of kaolin per ml of plasma. Ristocetin-induced platelet aggregating activity was determined as described (32), except that paraformaldehyde-fixed platelets were used at a concentration of about 80,000/mm3 and the volumes were reduced to 280 μl of platelet suspension. 35 μl of FVIII/vWF, and 35 μl of ristocetin (15 mg/ml) were added to the method of Head et al. (33), but 0.05% disodium EDTA was used in the Tris-glucose buffer and bovine serum albumin was omitted. The radioreceptor assay for the binding of native and alkylated FVIII/vWF to paraformaldehyde-fixed platelets was that of Kao et al. (32) except that 8 × 10^5 platelets per incubation were used. For this assay, FVIII/vWF was labeled with Na125I using insolubilized lactoperoxidase (29).

Alkylation of FVIII/vWF

In three separate series of alkylations, FVIII/vWF (1.6–1.9 mg/ml) was reduced with 0.1 mM to 25 mM DTT for 2.5 h at 37°C under nitrogen; control incubations of FVIII/vWF without DTT were also included. The buffer was 0.2 M ammonium formate-0.15 M NaCl-0.01 M Tris, pH 7.35, which had been deaerated and perfused with extra-dry nitrogen for 1 h before use. Alkylation of reduced FVIII/vWF was performed for 40 min in the dark at 24°C. [3H]iodoacetic acid dissolved in 0.5 M Tris-0.15 M NaCl, pH 7.4 was added to give a final concentration equal to that of DTT. The solution was then exhaustively dialyzed against 0.05 M sodium phosphate-0.15 M NaCl, pH 7.35. For quantitative alkylation, aliquots of 10–20 μl were counted in a liquid scintillation spectrometer (Model 3255, Packard Instrument Co., Inc., Downers Grove, IL). Each of the alkylated FVIII/vWF samples (1.0–1.4 mg/ml) was then divided and subjected to both FVIII and vWF functional assays as well as to electron microscopic and SDS gel electrophoretic analyses.

Electrophoretic Analysis

SDS 5% polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (34) or Laemmli (35). SDS-2% polyacrylamide-0.5% agarose gels were prepared as described by Peacock and Dingman (36), but were pre-electrophoresed overnight at room temperature at 40 V in 0.1% SDS-0.1 M sodium phosphate, pH 7.1. Samples in 0.1% SDS-0.1 M urea-0.01 M sodium phosphate, pH 7.1, were run for 30 min at 2 mA/gel and then at 4.5 mA/gel until the dye band reached the bottom. Electrophoresis was also performed in agarose according to the method of Fass (6), but using an agarose concentration of 1.5% and the Weber and Osborn sample and running buffers (34). A current of 1 mA/gel was used for the first 30 min and then 4 mA/gel for 3.5 h. The agarose gels were stained for 15 min at room temperature in 0.2% Coomassie Blue, 45% methanol, and 9% acetic acid, and then destained in 14% acetic acid-10% methanol. Human IgM and its multimers, polymerized according to the method of Fass (6), were used as high molecular weight standards for nonreduced samples and for FVIII/vWF alkylated after reduction by 0.1 mM DTT. Subunit molecular weights of reduced FVIII/vWF were estimated in SDS-agarose gels using the following reduced proteins as standards: thyroglobulin, ferritin, α1-macroglobulin, myosin, β-galactosidase, and phosphorylase B.

Electron Microscopy

Each FVIII/vWF sample, whether native or reduced and alkylated, was diluted to 30 μg/ml in 0.2 M ammonium formate or 0.2 M ammonium formate-0.1 M NaCl, pH 7.35, and mixed with one-half volume of glycerol. A 20-μl aliquot of this mixture was sprayed onto freshly cleaved mica, dried in vacuo, and rotary shadowed with platinum as previously described (26, 27, 37). Samples were examined in a Siemens Elmiskop 101 electron microscope at 80 kV accelerating voltage with a 50 μm objective aperture. Postmicrographs were taken at ×40,000 magnification and photographically enlarged to ×140,000 final magnification. 100 molecules were selected for length measurement if they were extended enough so that the contour could be traced with as little ambiguity as possible. Contour lengths of native FVIII/vWF and fibronectin were measured using a sonic digitizer (Graf-Pen; Scientific Accessories Corp., Southport, CT) coupled to a PDP-11/70 computer (Digital Equipment Corp., Marlboro, MA). Lysophilized fibronectin was dissolved in 0.2 M ammonium formate and prepared for microscopy as described previously (38). Many of the molecules in this preparation were fragmented, but intact molecules of normal length (~140 nm) could be found. For comparison, human fibrinogen and IgM samples were also prepared for electron microscopy essentially the same way as the FVIII/vWF.

RESULTS

Electron Microscopy of Native and Reduced FVIII/vWF

The electron microscopic structure of rotary shadowed native FVIII/vWF, presented in Fig. 1, resembles very flexible filaments with irregularly spaced small nodules and heterogeneous lengths. Other representative examples of native FVIII/vWF from several different preparations are shown in Fig. 2, which further illustrates the variety of their sizes and shapes. At this magnification, or higher, a repeating structure for the 200,000-dalton FVIII/vWF subunit was not readily discernible and the molecules lack fixed, large globular domains as seen in some proteins, such as fibrinogen and IgM (Fig. 2 G). The electron microscopic structure of vWF protein, which was separated from FVIII procoagulant activity by chromatography in 0.25 M CaCl2 (8), was indistinguishable from that of FVIII/vWF.

To evaluate the size distribution of FVIII/vWF, the contour lengths of 100 native molecules were measured. They ranged from 50 to 1,150 nm with a mean length of 478 nm, as shown in Fig. 3. The corresponding molecular weights were deter-
mined by assuming that the strands were cylinders of 2.5 nm diameter and packed with protein of partial specific volume 0.72 cm$^3$/g (7). This diameter was estimated by comparison to the similar strands of fibronectin, a simpler molecule that is well-characterized in terms of mass per unit length (38). When specimens of FVIII/vWF and fibronectin were prepared simultaneously using both unidirectional and rotary shadowing, the average diameter of the FVIII/vWF filament was ~25% larger than that of fibronectin, which had been established as 2 nm (38, 39). Using the lengths, the average width and the partial specific volume, the molecular weight of the average-length FVIII/vWF molecule was estimated to be $2 \times 10^6$ daltons with the standard deviation being $0.9 \times 10^6$ daltons. When calculated by this method, the molecules shown in Fig. 2A ranged in molecular weight from $1.1 \times 10^6$ to $4.8 \times 10^6$ daltons. It should be noted that there are some straight segments of the vWF strand, of length 35 nm (Fig. 1), that are remarkably similar in appearance to fibronectin. Since the fibronectin subunit is 70-80 nm long, the 35 nm straight segments seen here may correspond to one-half of the 200,000-dalton vWF subunit.

The size and shape of the FVIII/vWF molecules were dramatically altered by progressive reduction and alkylation of disulfide bonds. As shown in Fig. 2B, alkylation after incubation of FVIII/vWF with 0.1 mM DTT in dilute neutral buffer resulted in slightly shorter filaments containing some large nodules. Reduction with 0.5 mM DTT and alkylation transformed the structure of FVIII/vWF into very large nodules that were often connected by short filamentous segments (Fig. 2C). There were as many as four nodules per molecule and the dimeric structure appeared frequently. The corrected nodule diameters were 12-18 nm and were derived from the measured diameters by subtracting 4 nm, which represents the estimated 2 nm-thick platinum shell around the molecule (26, 40). At higher concentrations of DTT the structure remained nodular, but the filamentous connections were no longer evident. Some heterogeneity in nodule size and in the tendency to aggregate was seen at 2 mM and 10 mM DTT (Fig. 2D and E, respectively); however, the trend was toward a single-cell structure of uniform diameter (~10-11 nm) such as shown in Fig. 2F for FVIII/vWF alkylation after reduction with 25 mM DTT. Assuming these nodules are spherical and using a partial specific volume of 0.72, the maximum molecular weight of this reduced FVIII/vWF form was estimated between 430,000 and 580,000 daltons. Other experiments in which FVIII/vWF was analyzed immediately after reduction at the above DTT concentrations, but not alkylation, showed the same effects on structure and thereby exclude the possibility that alkylation caused the molecular changes.

Fig. 2G shows the rotary shadowed electron microscopic structure of some other large plasma proteins for comparison with FVIII/vWF: the familiar trinodular structure of fibrinogen (340,000 daltons) (26) in the upper two panels; the uniformly thin, filamentous structure of fibronectin (440,000 daltons) (38) in the middle panels; and the compact penta- and hexanodular structure of IgM (800,000-950,000 daltons) (41) at the bottom.

Electrophoretic Analyses of Native, Reduced, and Alkylated FVIII/vWF

Figure 4a shows the effect of reduction on the structure of FVIII/vWF as observed by electrophoresis in SDS-1.5% agarose. In the absence of reducing reagents FVIII/vWF was multimeric with eight bands ranging in molecular weight from $0.8 \times 10^6$ to $7 \times 10^6$ daltons (Fig. 4a, lane 1), using crosslinked IgM as the standard. The multimeric forms seen in gels 2-6 are those of the partially reduced and alkylated FVIII/vWF samples for which the electron microscopic structure was shown in Fig. 2, Panels B-F. At 0.1 mM DTT the predominant bands had apparent molecular weights of $0.8 \times 10^6$, $1.2 \times 10^6$ and $1.6 \times 10^6$ daltons (Fig. 4a, lane 2). The actual molecular weights...
of these multimers are probably 50–60% of these apparent values (see reference 6), because it is known that crosslinked IgM, like other crosslinked proteins, is not an ideal molecular weight standard, especially for comparison with partially reduced proteins. Unfortunately there are no standards for reduced proteins with such high molecular weights. Further reduction and alklylation sharply diminished the number of higher molecular weight forms. The dominant band resulting from reduction at DTT concentrations of 2 mM or greater (Fig. 4a, lanes 4–6) had the same mobility as fully reduced, nonalkylated FVIII/vWF (Fig. 4a, lane 7). The apparent molecular weight of this band when determined in comparison to reduced standards in SDS-agarose gels was 350,000 daltons; however, when determined either by Weber-Osborn SDS-polyacrylamide gel electrophoresis or by ultracentrifugation (7), the molecular weight was 200,000 daltons and corresponded to the monomeric subunit of FVIII/vWF. The faint band above the monomer in lanes 4–6 (Fig. 4a) and the major band in gel 3 (0.5 mM DTT) correspond to a dimer of the FVIII/vWF subunit.

The FVIII/vWF samples that had been progressively reduced and then alkylated barely penetrated SDS-5% polyacrylamide gels when analyzed by standard methods (34, 35). As shown in Fig. 4b, when these same samples were further reduced by heating for 5 min at 90°C with 1 M 2-mercaptoethanol in 6 M urea-0.1% SDS, the multimeric structure was reduced to a single band which migrated well into the gel, but the mobility of each was inversely proportional to the extent of
prior alkylation. Thus reduced samples of FVIII/vWF that had 28%, 51%, and 73% of its disulfide bonds alkylated (in Fig. 4b, lanes 1, 2, and 3, respectively) appeared increasingly larger than fully reduced, nonalkylated FVIII/vWF (Fig. 4b, lane 4), which had an apparent molecular weight of 200,000 daltons. This effect may be due to decreased binding of SDS as a result of the added carboxymethyl groups (42).

As shown in Fig. 4c, analysis of FVIII/vWF by SDS-2% polyacrylamide-0.5% agarose gel electrophoresis gave improved resolution of its multimeric structure compared to that observed on SDS-1.5% agarose gels. As on SDS-agarose gels, the various multimers of partially reduced and alkylated FVIII/vWF penetrated these gels without further reduction, but their mobility decreased with increasing degree of alkylation (Fig. 4c, lanes 3 and 4). In view of these effects of alkylation on the mobility of protein-SDS complexes in polyacrylamide gel electrophoretic systems, the two major bands of FVIII/vWF alkylated at 25 mM DTT (Fig. 4c, lane 4) must be the monomer and dimer of the 200,000-dalton subunit since:

(a) their apparent molecular weights differ by a factor of two; and
(b) the mobility of the lowest band (on either Fig. 4c, lane 3 or 4), when corrected for the effect of alkylation, is the same as that of the fully reduced, nonalkylated FVIII/vWF 200,000-dalton subunit. Thus FVIII/vWF reduced with 2 mM DTT or more exists primarily in the monomeric and dimeric forms (see Fig. 2D–F and Fig. 4a, lanes 4–6), whereas treatment with 0.5 mM DTT or less produced only dimers and higher multimers of the 200,000-dalton subunit.

The multimeric structure of nonreduced FVIII/vWF appears in gel 1 of Fig. 4c as a series of 12 paired bands with an upper apparent molecular weight of $5.2 \times 10^6$. This progression of doublet bands, which was also discernible to a lesser extent on SDS-1.5% agarose gels, is characterized in Table I for the major multimers. The third column of Table I shows that the spacing within the doublets is about 280,000 daltons, whereas the spacing between them is 480,000 daltons; these probably represent increments of one and two subunits. The number of subunits in each multimer was estimated by dividing its apparent molecular weight in this electrophoretic system, given in the second column of Table I, by the apparent molecular weight per subunit, which in case 1 was assumed to be the actual weight of 200,000 daltons and in case 2 was estimated from the apparent molecular weight intervals between multimer bands (third column, Table I) to be 250,000 daltons.

| Band number| Apparent mol wt $\times 10^6$ | Number of subunits$\dagger$ |
|------------|-----------------|-----------------|
| 1          | 1.2             | 6               |
| 2          | 1.5             | 7-8             |
| 3          | 2.0             | 10              |
| 4          | 2.3             | 11-12           |
| 5          | 2.8             | 14              |
| 6          | 3.0             | 15              |
| 7          | 3.4             | 17              |
| 8          | 3.7             | 19              |

* Determined by SDS-2% polyacrylamide-0.5% agarose gel electrophoresis using cross-linked IgM multimers as the molecular weight standards.

$\dagger$ Designated bands are shown in gel 1 of Fig. 4c.

$\dagger$ Determined from column 2 using a subunit apparent molecular weight of 200,000 daltons for case 1 and 250,000 daltons for case 2.
Ristocetin-induced Platelet Aggregating Activity of Reduced and Alkylated FVIII/vWF

This activity, which in Fig. 5 we arbitrarily equate with vWF activity, was progressively diminished as disulfide bonds were reduced. FVIII/vWF alkylated after reduction with 0.1 mM DTT retained 72% of the vWF specific activity of the nonreduced control, while FVIII/vWF samples alkylated after reduction with 2 mM and 10 mM DTT had only 38% and 17% as much, respectively. The reducing agent 2-mercaptoethanol was 10-15 times less effective than DTT; for example, treatment with 5 mM 2-mercaptoethanol as compared to only 0.35 mM DTT, with or without alkylation, was required to decrease the vWF specific activity by 50%.

The number of disulfide bonds reduced at each DTT concentration was estimated by alkylation using [3H]iodoacetic acid, since purified FVIII/vWF, whether denatured or not, contains no detectable free sulfhydryl groups (43). Fig. 5 shows that in the absence of denaturing agents, the maximum number of moles of [3H]acetate incorporated per mole of FVIII/vWF 200,000-dalton subunit during alkylation was 7.4 μM. The incorporation of 10 mol of [3H]acetate per mole of FVIII/vWF subunit corresponded to 4.6 x 10^6 cpm/mg of FVIII/vWF protein.

Binding of Reduced and Alkylated FVIII/vWF to Platelets

The effect of reduction of FVIII/vWF on its platelet binding in the presence of ristocetin was assessed by competitive displacement of 125I-FVIII/vWF by serial dilutions of native or reduced and alkylated FVIII/vWF. Fig. 6 demonstrates that nonreduced FVIII/vWF decreased the specific binding of 125I-FVIII/vWF by 50% was 0.7 μg compared with 1.7 μg of the reduced protein. Further alkylation of FVIII/vWF at 0.5 mM and 2.0 mM DTT had little additional effect on its specific platelet binding capability; in each case 50% specific binding was obtained with 2.0 μg of the alkylated FVIII/vWF. However, FVIII/vWF specimens that were alkylated at DTT concentrations of 10 mM and 25 mM were considerably less competitive, with 50% specific binding occurring at 3.7 μg and 8.7 μg, respectively.

There was a very good correlation (r = 0.98) between specific platelet binding and the vWF activity of native or alkylated FVIII/vWF (Fig. 7). The initial rate of ristocetin-induced platelet aggregation was plotted as a function of the percent specific binding of 125I-FVIII/vWF displaced by unlabeled native or reduced and alkylated FVIII/vWF. Native FVIII/vWF and each reduced species had essentially the same vWF activity when measured at the different protein concentrations required to displace 50% of the 125I-FVIII/vWF platelet binding. The nonzero y-intercept in Fig. 7 may reflect a greater sensitivity of the ristocetin-induced platelet aggregation measurement.
creased above 0.1 mM. The activity was only 50% at 1 mM corresponding to even the largest molecules, which are about 12-18 nm in diameter, that an average of only five disulfide bonds were alkylated, the size and number of nodules on the filaments were increased and only species smaller than 1.6 million daltons were observed by SDS-agarose electrophoresis. Our findings that reduction of FVIII/vWF with 0.5 mM DTT gives large nodular structures of 12-18 nm in diameter, that an average of only five disulfide bonds per 200,000-dalton subunit are reduced by this DTT concentration, and that the predominant species on SDS-agarose gels is the dimer, indicate that only a few interchain disulfide bonds are responsible for maintaining the filamentous structure of the native FVIII/vWF molecule. With the reduction of 21 disulfide bonds per subunit at 2 mM DTT, the nodules become considerably more compact (10-11 nm diameter) and the predominant band on SDS-agarose gels is the monomeric subunit. Whereas limited interchain disulfide bond reduction accounts for the formation of the globular structures at the lowest DTT levels, reduction of intrachain disulfide bonds at the higher DTT concentrations may cause the globules to become more compact by increasing the potential for hydrophobic interactions. Yet we observed that even when half of the 62 disulfide bonds per 200,000-dalton subunit were reduced, as occurs at 10 mM DTT, the volume of the resulting single nodules can still accommodate up to 500,000 daltons of a compact globular protein. It is likely, however, that the structure of the collapsed vWF filament obtained after reduction in non-denaturing buffers is more open and loosely packed than that of an intact globular protein. In this case each nodule may, as suggested by the agarose gel-electrophoretic analyses, correspond to a single 200,000-dalton subunit. These changes in electron microscopic structure are more dramatic than those usually seen when a protein is progressively reduced. Reduction of fibronectin, for example, results in strands half the length of the native dimeric molecule; the structure of these strands is, however, identical to that of the unreduced molecules (H. P. Erickson, unpublished observations).

As estimated from electron microscopy (Fig. 3), the range and frequency distribution of molecular weights of our nonreduced FVIII/vWF are in good agreement with those determined by SDS-2% polyacrylamide-0.5% agarose gel electrophoresis. By either technique, the largest multimer was 5 × 10^6 daltons and the most prominent species was 2 × 10^6 daltons. This molecular weight distribution is characteristic for FVIII/vWF that is purified, as ours is, from intermediate-purity concentrates. For such preparations, Weinstein and Deykin (44) determined a molecular weight range of 1.4-5 × 10^6 daltons by SDS-gel electrophoresis. Human cryoprecipitate, however, is reported to have FVIII/vWF species of molecular weight up to 10 × 10^6 daltons by gel filtration or SDS-agarose gel electrophoresis (44-46). Importantly this distribution of multimer size overlaps significantly with that of our FVIII/vWF preparations (21, 44). Whether or not the largest FVIII/vWF forms actually circulate in vivo remains controversial. Some investigators have concluded from SDS-gel electrophoresis of fresh plasma, followed by heterologous antibody labeling, that FVIII/vWF forms as large as 10-15 million daltons are present in plasma (45, 47). Others, however, have found by gel filtration that freshly drawn warm plasma does not contain the large forms found in cryoprecipitate (18, 48). In any case, our preliminary electron micrographs of the earliest fractions with FVIII/vWF activity from Sephacryl S-1000 gel filtration of cryoprecipitate reveal FVIII/vWF forms that are essentially identical to those shown here, except that some are less folded. As FVIII/vWF is titrated with the reducing agent DTT, there are striking changes in its structure as seen by electron microscopy. At the lowest DTT concentration tested, when fewer than 0.5% of the disulfide bonds were alkylated, the size and number of nodules on the filaments were increased and only species smaller than 1.6 million daltons were observed by SDS-agarose electrophoresis. Our findings that reduction of FVIII/vWF with 0.5 mM DTT gives large nodular structures of 12-18 nm in diameter, that an average of only five disulfide bonds per 200,000-dalton subunit are reduced by this DTT concentration, and that the predominant species on SDS-agarose gels is the dimer, indicate that only a few interchain disulfide bonds are responsible for maintaining the filamentous structure of the native FVIII/vWF molecule. With the reduction of 21 disulfide bonds per subunit at 2 mM DTT, the nodules become considerably more compact (10-11 nm diameter) and the predominant band on SDS-agarose gels is the monomeric subunit. Whereas limited interchain disulfide bond reduction accounts for the formation of the globular structures at the lowest DTT levels, reduction of intrachain disulfide bonds at the higher DTT concentrations may cause the globules to become more compact by increasing the potential for hydrophobic interactions. Yet we observed that even when half of the 62 disulfide bonds per 200,000-dalton subunit were reduced, as occurs at 10 mM DTT, the volume of the resulting single nodules can still accommodate up to 500,000 daltons of a compact globular protein. It is likely, however, that the structure of the collapsed vWF filament obtained after reduction in non-denaturing buffers is more open and loosely packed than that of an intact globular protein. In this case each nodule may, as suggested by the agarose gel-electrophoretic analyses, correspond to a single 200,000-dalton subunit. These changes in electron microscopic structure are more dramatic than those usually seen when a protein is progressively reduced. Reduction of fibronectin, for example, results in strands half the length of the native dimeric molecule: the structure of these strands is, however, identical to that of the unreduced molecules (H. P. Erickson, unpublished observations).

Of the three FVIII/vWF functions tested, the most sensitive to structural alterations was its binding to platelets in the presence of ristocetin. The specific binding of FVIII/vWF alkylated at 0.1 mM DTT dropped to 40% of the control level when based on the microgram amount required to displace 50% of the ^125I-FVIII/vWF (Fig. 6). On a molar basis, however, only 16% binding remained, because at this stage of reduction
the mean multimer size of FVIII/vWF as estimated by SDS gel electrophoresis was four subunits compared with 10 for that of nonreduced FVIII/vWF. Platelet binding of the FVIII/vWF dimer, which appears on SDS gels as the major product of reduction by 0.5 mM DTT, was ~35% of native FVIII/vWF on a weight basis or 7% on a molar basis. The loss of ristocetin-induced platelet aggregating activity in reduced FVIII/vWF (Fig. 5) was equal to the diminished platelet binding except at the lowest DTT concentration tested, for which case the platelet aggregating activity was disproportionately higher than the platelet binding. Perhaps the increased size of the nodular regions in the filamentous structure of FVIII/vWF at 0.1 mM DTT enhanced the platelet agglutinating effectiveness of those FVIII/vWF molecules that could still bind to the platelet receptor. Of interest in this respect are the previous reports of transitory enhancement of FVIII/vWF platelet aggregating activity after very limited reduction (16, 19). In comparison with activity losses caused by removal of carbohydrate (11, 12), FVIII/vWF alkylated after reduction with 0.1 mM DTT had platelet binding similar to asialo-FVIII/vWF and slightly less vWF activity per mole. Our losses in ristocetin-induced platelet aggregating activity and platelet binding capacity of FVIII/vWF with progressive reduction are similar in magnitude to those recently reported (20). Moreover, the functional losses we observed coincided with the very earliest stage in the transformation of filamentous FVIII/vWF multimers to globular forms.

In contrast to the platelet related activities, FVIII procoagulant activity persisted during these structural changes (Fig. 8); however, it was not immutable to reduction as others have described (5, 16, 17, 19, 20). A 50% loss of FVIII activity occurred at 30 mM 2-mercaptoethanol or at 1 mM DTT; in either case FVIII/vWF had no fibrillar structures remaining and was composed mainly of the 400,000-dalton dimer. Whether this residual activity was stable beyond several hours in the absence of the larger vWF multimers was not specifically tested. Because the FVIII procoagulant moiety contributes <1% of protein content of the FVIII/vWF complex, the measured incorporation of [14C]acetate after reduction of FVIII/vWF (Fig. 5) does not indicate to what extent FVIII itself was alkylated. The final 34% of the FVIII procoagulant activity was lost between 2 mM and 50 mM DTT when there were no further changes in FVIII/vWF structure, as assessed by electron microscopy and SDS-agarose gel electrophoresis. This suggests that alklylation of the FVIII entity may contribute to the loss of procoagulant activity.

At this time we are unable to be exact about the assembly of FVIII/vWF into multimers. The remarkable structure of a thin filament of mostly uniform diameter indicates that it cannot be a random polypeptide chain, but instead must be coiled or folded into an elongated structure that propagates along its long axis to give the well defined filaments we observe. The precise structural model for FVIII/vWF must be consistent with several additional observations from our work and that of others which suggest: (a) The basic unit, or protomer, contains two 200,000-dalton subunits joined by disulfide bonds that are relatively resistant to reduction in nondenaturing solvents. Besides data shown here, evidence for such a dimeric protomer has been given by Counts et al. (5) and others (44, 49), although some have suggested a tetrameric protomer (45, 47, 50). (b): The disulfide bonds that link protomers into a strand are very susceptible to reduction in dilute neutral buffers. (c): There is a recurrent nonuniform difference in molecular weight of 250,000 daltons alternating with 500,000 daltons as the multimers increase in length (Table I). This is evident as a series of doublet bands on SDS-gel electrophoresis (Fig. 4c, lane 1) and has been noted for FVIII/vWF from intermediate-purity concentrates, but not from cryoprecipitate (44). However, a similar nonuniformity was recently reported for FVIII/vWF in fresh normal plasma and did not appear due to proteolysis (46). (d): Because a protomer of 400,000 daltons is released very early in the course of reduction, before any 200,000-dalton subunits are seen, a model in which each protomer is disulfide-linked with a monomeric subunit in an alternating progression is not possible. Perhaps the vWF multimers are composed of subunits that differ only in the number and position of their disulfide bonds, such as to allow for two different conformational states; this may then account for the alternating spacing observed between adjacent bands on SDS gels of nonreduced FVIII/vWF (Table I).

Just as with other proteins, we assume that the process of FVIII/vWF absorption to a mica surface gives a distribution of molecular shapes similar to that present in solution. To this extent it is of interest that FVIII/vWF may be the largest circulating protein, but unlike other plasma macromolecules such as IgM, α2-macroglobulin and fibrinogen, it has no prominent globular domains. It does bear structural similarity to the fibrillar protein, fibroneciton, which as a uniform length and a molecular weight of 440,000 and like FVIII/vWF, is synthesized by the endothelial cell (38, 39). Some have postulated that filamentous proteins are well-suited for bridging the distances between binding sites located on cell surfaces, Type IV collagen and matrix components of basement membranes (38, 51); however, the detailed steric arrangements of such multicomplexes remain unknown. We speculate that the long fibrillar FVIII/vWF multimers span the distances between the vascular intimal surface and platelets as the latter flow by an area of injury. In this way the endothelial cell and several platelets can be brought together to form a platelet mass that seals an injured vessel. The FVIII/vWF fibrillar multimers in plasma may collect at a site of vascular damage or simply become exposed as a consequence of injury to their cell of origin, the endothelial cell. Whether the small forms of FVIII/vWF produced by partial reduction are analogous to the small multimers with decreased function in Type IIA von Willebrand's disease (1) is being investigated.

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