Stoichiometry of the Porcine Factor VIII-von Willebrand Factor Association*

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Fletcher VIII and von Willebrand factor (vWF) are glycoproteins that form a tightly bound complex in plasma. The interaction of porcine factor VIII with porcine vWF was studied by analytical velocity sedimentation. A single ~240-kDa species of factor VIII was isolated for use in the analysis. In contrast, when analyzed by agarose/sodium dodecyl sulfate-polyacrylamide gel electrophoresis, vWF consisted of a population of >10 multimers derived from a 270-kDa monomer. A single boundary (s20,w = 7.2 S) was observed during velocity sedimentation of factor VIII at 260,000 × g. A single boundary also was observed for vWF (weight-average s20,w = 21 S) at 42,000 × g. Under conditions of excess factor VIII, the weight-average s20,w of the factor VIII-vWF complex was 40 S at 42,000 × g. At 260,000 × g, the factor VIII-vWF complex had sedimented completely, leaving only free factor VIII. The height of the plateau region of the factor VIII sedimentation velocity curve at 260,000 × g was studied as a function of several starting concentrations of vWF. The experiments were done under conditions in which the effect of radial dilution was negligible so that the plateau height was a measure of the concentration of free factor VIII. The plateau height decreased linearly as the concentration of vWF was increased, indicating that the association was essentially irreversible under the conditions used. A stoichiometry of 1.2 vWF monomers/factor VIII molecule was calculated from the slope of the line. Assuming one factor VIII-binding site/vWF monomer, these results indicate that all factor VIII-binding sites are accessible in the vWF multimer.

Factor VIII and von Willebrand factor (vWF) are glycoproteins that are necessary for normal hemostasis. Their corresponding deficiency states are known as hemophilia A and von Willebrand's disease, respectively. When activated by proteolytic cleavage, factor VIII is a cofactor for factor IXa in the activation of factor X in the intrinsic pathway of blood coagulation (1). Although factor VIII is synthesized as a single 267-kDa polypeptide chain (2), it is isolated from plasma as a heterogeneous population because of proteolysis of the parent molecule (3-11). The population consists of heterodimers that contain an invariant light chain noncovalently linked to a variably sized heavy chain (4, 10).

von Willebrand factor circulates as a population of multimers (12). The monomeric subunit is synthesized in endothelial cells (13) and megakaryocytes (14) as a single polypeptide chain with a molecular mass of 270 kDa (15). Multimers are assembled in the cell of origin by linking subunits via disulfide bonds (16). The size range of porcine vWF has been estimated to be 1-20 MDa by agarose/SDS gel electrophoresis (12). von Willebrand factor participates in platelet-vasculat vessel adhesion and perhaps platelet aggregation through interactions with collagen, non-collagen vessel wall components, and platelet surface glycoproteins (17). Additionally, vWF binds factor VIII to form a tight, noncovalently linked complex (18). This interaction prolongs the half-life of factor VIII in plasma (7).

The binding capacity of native, multimeric vWF for factor VIII is unknown. We have isolated a single ~240-kDa species of porcine factor VIII and determined its extinction coefficient by far-UV absorbance spectroscopy. Additionally, the extinction coefficient of porcine vWF has been determined by dry weight measurement and differential refractometry (with equivalent results). Using these rigorously defined preparations, the factor VIII-vWF interaction has been analyzed by velocity sedimentation. The strength of the interaction coupled with the large difference in size between factor VIII and vWF or the factor VIII-vWF complex makes it possible to distinguish free factor VIII from factor VIII in complex. We have used this method to measure the stoichiometry of the interaction of factor VIII with multimeric vWF.

EXPERIMENTAL PROCEDURES

Electrophoresis—Discontinuous SDS-PAGE was done using the buffer system of Laemmli (19). Samples containing 1% (v/v) SDS with or without 1-2% (v/v) β-mercaptoethanol were heated for 2-5 min in a heating block maintained at 100 °C. Factor VIII was analyzed by electrophoresis of nonreduced samples in 7% acrylamide gels. Reduced vWF was analyzed by electrophoresis in 5% acrylamide gels. Myosin (290 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and chymotrypsinogen (28 kDa) (Bethesda Research Laboratories) were used as standards. Additionally, human fibrinogen (340 kDa), purified according to the method of Straughn and Wagner (20), was used as a standard in gels containing vWF. Agarose/SDS-PAGE of vWF was done as described by Chopek et al. (15). Serum from a patient with Waldenström's macroglobulinemia was used to identify immunoglobulin M as a molecular mass marker (1 MDa) (12). Factor VIII was detected by silver staining (21). vWF was detected by staining with Coomassie Blue R-50.

Factor VIII and von Willebrand Factor Assays—Factor VIII and thrombin-activated factor VIII were measured by one- and two-stage assays, respectively, using activated partial thromboplastin and hu-
man factor VIII-deficient plasma (George King Biomedical) (4). One unit of porcine factor VIII was defined as the amount of factor VIII in 1 ml of citrated normal porcine plasma.

Porcine von Willebrand factor was measured in an assay based on its ability to agglutinate fixed human platelets in the presence of ristocetin (Bio/Data Corp., Hatboro, PA) (22). One unit of porcine von Willebrand factor was defined as the amount in 1 ml of citrated normal porcine plasma.

Isolation of Proteins—Commercial porcine factor VIII concentrate (Porton Products) was dissolved by adding 4 ml of 0.1 M MES, 0.1 M NaCl, 0.255 M MgCl₂, pH 6.0, to each of 10 bottles of concentrate at room temperature to dissociate factor VIII from vWF. Each bottle contained approximately 8 mg of total protein and 60 units of factor VIII. Porcine factor VIII was isolated from commercial concentrate by murine monoclonal anti-porcine factor VIII-Sepharose chromatography. The hybridoma cell line producing the antibody was generously provided by Dr. D. N. Fass (Mayo Clinic, Rochester, MN).

Three heterodimers of porcine factor VIII having apparent molecular masses by SDS-PAGE of 160 + 76, 130 + 76, and 82 + 76 kDa have been described previously (4, 23). The 76-kDa chain represents a common light chain, whereas the three heaviest chains have a common NH₂-terminal sequence (2) and are derived from variable pro- teins of the middle region, or B domain (24), of the parent single-chain factor VIII molecule. Two of the four commercial concentrate that have been examined have lacked the 130-kDa heavy chain species. The 160 + 76 (or ~240-kDa) heterodimer was isolated from a lot lacking the 130-kDa species by cation-exchange HPLC using an HR 5/5 Mono S column (Pharamacia LKB Biotechnology Inc.) (2.5 × 85-cm column) gel permeation chromatography and concentrated using conditions described previously for human vWF (25). The final preparation, approximately 1 mg/ml in 2 ml of 0.15 M NaCl, 0.02 M Tris-Cl, pH 7.4 (TBS), was stored at 4 °C. The final product had a specific activity of 150 units/mg and was used within 48 h of preparation. On SDS-PAGE using reducing conditions, greater than 90% of the preparation migrated as a single band at ~250 kDa. On composite agarose/SDS-PAGE using nonreducing conditions, approximately 10 bands, corresponding to vWF multimers, were identified. Additionally, the largest forms were not resolved into discrete bands. The smallest band had an apparent molecular mass of approximately 1 MDa since it migrated slightly slower than immunoglobulin M.

Extinction Coefficients—The extinction coefficient at 280 nm of the ~240-kDa factor VIII heterodimers was determined in factor VIII spectrophotometry as described by van Lersel et al. (26). Samples were centrifuge buffer exchange into 0.1 M sodium phosphate, pH 6.5, by chromatography on a Sephadex G-25 superfine column (Pharmacia LKB Biotechnology Inc.) before analysis. Determinations were done in duplicate from samples from a single preparation. The extinction coefficient at 280 nm of vWF was determined in duplicate on 20 prepara-

RESULTS AND DISCUSSION

Velocity Sedimentation of Factor VIII and vWF—We isolated the largest heterodimer of porcine factor VIII (~240 kDa) by cation-exchange nondialyzing HPLC in sufficient quantity for study by analytical ultracentrifugation (Fig. 1). This represents the first reported isolation procedure for a homogeneous preparation of factor VIII. The lack of hetero-
genre simplifies the analysis of the interaction of factor VIII with vWF. A single band was observed during velocity sedimentation of factor VIII (Fig. 2), which is consistent with a single sedimenting species. The sedimentation coefficient obtained from linear regression analysis of the plot shown in Fig. 2 (inset) was 7.2 S. The same value (within 0.2 S) was obtained when the concentration of factor VIII was decreased 4-fold, indicating that there was no concentration dependence of the sedimentation coefficient under the conditions used in this study.

Despite its known heterogeneity, only a single band was seen during velocity sedimentation of vWF (Fig. 3). This presumably represents overlapping boundaries due to the size distribution of multimers over regularly spaced intervals (12). Heterogeneity is also indicated from the comparison of the sedimentation coefficients calculated by both midpoint and second moment analysis. The two methods gave different values (Table 1), which is consistent with heterogeneity (31).

The sedimentation coefficient of vWF was not significantly different at a 4-fold lower concentration, indicating that it has no concentration dependence under the conditions used in this study (not shown). Therefore, the value in the Table 1 represents the weight-average 〈s²〉 of the sample. vWF was purified by gel permeation chromatography. Agarose/SDS-PAGE of various fractions from this step showed that the distribution of the population of multimers shifted to larger

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FIG. 1. SDS-PAGE of the ~240-kDa porcine factor VIII heterodimer. A nonreduced sample (1.0 μg) of factor VIII, obtained by Mono S HPLC, underwent electrophoresis in 7% polyacrylamide, followed by silver staining as described under "Experimental Procedures." Also shown are the positions of the molecular mass markers in kilodaltons.

FIG. 2. Velocity sedimentation of the ~240-kDa factor VIII heterodimer. Factor VIII (0.09 mg/ml in Buffer A + 0.3 M NaCl underwent analytical velocity sedimentation at 260,000 × g as described under "Experimental Procedures." Five scans that were obtained at 4-min intervals are superimposed in the graph. The inset shows the logarithm of the boundary position, obtained by second moment calculations, plotted versus time.

Multimers as the elution volume decreased; as described previously (32). Since a fraction of vWF was not pooled (~20% by assay) to avoid lower molecular mass contaminants, the weight-average sedimentation coefficient obtained from this preparation may be higher than that of circulating vWF.

Previous velocity sedimentation comparing purified and plasma porcine vWF in sucrose density gradients yielded similar results; the estimated sedimentation coefficient is 25–32 S (33). The porcine vWF used in this study has similar properties since: 1) more than 10 multimers with apparent molecular mass >1 MDa were observed after electrophoresis in agarose/SDS-PAGE (not shown); and 2) the weight-average sedimentation coefficient of the vWF was 21 S (Table I).

Velocity Sedimentation of the Factor VIII-vWF Complex—

The ~240-kDa factor VIII heterodimer was mixed with vWF as described under "Experimental Procedures," and the resulting solution was analyzed by ultracentrifugation at 42,000 × g. The results for one concentration of vWF are shown in Fig. 4. The absorbance near the meniscus (~6.5 cm) is greater than zero due to free factor VIII. The weight-average sedimentation coefficient, calculated from second moment analysis after subtracting the base line due to free factor VIII, was 39 S. By midpoint analysis, the sedimentation coefficient was 48 S, again indicating heterogeneity of the sample (Table I).

The experiment was repeated at several concentrations of vWF with the factor VIII concentration held constant. The calculated sedimentation coefficients did not vary appreciably with concentration (Table I). This indicates that the experiments were done under conditions of excess factor VIII such that vWF was completely saturated.

Stoichiometry of the Factor VIII-vWF Interaction—After each experiment shown in the Table I, the relative centrifugal field was increased to 260,000 × g. Because of its size, the factor VIII-vWF complex had sedimented completely by the time the rotor had reached the target speed, which allowed the analysis of free (unbound) factor VIII. The plateau height of the sedimentation velocity curve yields the concentration of free factor VIII when calculated as described under "Experimental Procedures." The plateau height decreased as the vWF concentration increased due to the incorporation of factor VIII into the complex, but there was no detectable change in the sedimentation coefficient of factor VIII. The
The concentrations of factor VIII and vWF that are required for the calculation must be known accurately. This was done for factor VIII using far-UV spectroscopy. Because of its large size, solutions of vWF are turbid. Solution turbidity increases with decreasing wavelength, which precluded the use of this spectroscopic technique. Instead, determinations were done by both differential refractometry and dry weight determination with similar results. The extinction coefficient at 280 nm was then calculated after correcting for turbidity.

It is clear from inspecting the data in Table I that the factor VIII-vWF complex has significantly different hydrodynamic properties than free vWF. The sedimentation coefficient depends on several factors including the molecular mass and frictional coefficient of the sedimenting species. The latter depends on shape and degree of hydration. At a fixed frictional coefficient, the sedimentation coefficient, $s$, of a single species varies as $s \propto m^{2/3}$, where $m$ is the molecular mass (31). For heterogeneous substances such as vWF and the factor VIII-vWF complex, it is not strictly valid to calculate the sedimentation coefficient given an average molecular weight. Nevertheless, it is interesting to do the calculation as a rough estimate. Given a sedimentation coefficient of 21 S and a 1:1 stoichiometry for the interaction of factor VIII with the vWF monomer, the calculated sedimentation coefficient for the complex is 32 S. This is lower than the observed value of 40 S and indicates that the complex may be influenced by a relatively smaller frictional drag than is free vWF. This could be due to tighter coiling of the vWF multimer when it binds factor VIII.

In Fig. 6, a scale model of the completely saturated factor VIII-vWF complex is proposed. The structure of vWF is based on the work of Fretto et al. (35) and Fowler et al. (32). Their ultrastructural studies indicate that the vWF monomer is a fibrillar structure 60 nm long consisting of an NH$_2$-terminal G domain and a COOH-terminal R domain. The monomers are arranged -GR-RG- to form multimers. The vWF monomer is depicted as two cylinders placed end-to-end. The thick cylinder corresponds to the G domain, and the thin cylinder corresponds to the R domain. The radii of the anhydrous cylinders were calculated to be 1.6 and 0.85 nm for G and R, respectively, using published values for their molecular weights and partial specific volumes (35). The C-terminal ends of the monomer are linked to form the so-called vWF protomer (Fig. 6, lower). Factor VIII is shown as two spheres corresponding to the 76- and 166-kDa chains. Their radii were calculated to be 2.8 and 3.6 nm, respectively, assuming a partial specific volume of 0.72 mg/g. Factor VIII binds within 273 residues of the NH$_2$-terminal end of the 2050-residue vWF monomer (36), perhaps via the 76-kDa chain (37). In Fig. 6 (upper), a 2.2-MDa vWF multimer is shown with 8 bound factor VIII molecules. Although the vWF monomer...
may be rigid and elongated, electron microscopic and light scattering studies indicate that most multimers are supercoiled in solution instead of arranged linearly as in Fig. 6 (38, 39). If we assume that there is one binding site for factor VIII/vWF monomer, the results of this study are consistent with the hypothesis that all potential binding sites are accessible in native vWF.

If there is one potential binding site for factor VIII for each vWF monomer, it is interesting to ask how saturated vWF is in vivo. The reported range of specific activities of human factor VIII is 2300–8000 units/mg (5–11). At 1 unit/ml, this corresponds to 0.1–0.4 µg/ml. The concentration of human vWF has been estimated to be 5–10 µg/ml (18). According to these numbers, human vWF is 1–8% saturated with factor VIII. Recently, we found from the specific activity calculations and also from direct radioimmunoassay that the plasma concentration of porcine factor VIII is 2–3 µg/ml, which is considerably higher than that for human factor VIII. The concentration of porcine vWF, calculated from the specific activity of the purified material from this or previous (40, 41) studies, is estimated to be 4–8 µg/ml. Thus, porcine vWF may be >50% saturated with factor VIII. At sites of hemostasis, it is conceivable that the local concentration of factor VIII increases significantly relative to vWF, thereby leading to increased saturation of vWF. It is possible that factor VIII might regulate vWF function under these conditions.

Velocity sedimentation offers several advantages as a technique to study the interaction of factor VIII with vWF. The factor VIII-vWF equilibrium is essentially irreversible under the conditions used in this study. With excess factor VIII, this results in the formation of only two species: factor VIII and the completely saturated complex. Since the species differ markedly in sedimentation properties (S vs versus 40 S), they can be differentiated easily. Thus, the technique yields quantitative information about the interaction in solution. Additionally, the use of the photoelectric scanner interfaced to a microcomputer allows the measurement of the concentration and sedimentation coefficient of less than 10 µg of factor VIII, vWF, or the factor VIII-vWF complex in approximately 1 h. This technique may be useful in the study of other aspects of the interaction of factor VIII with vWF, such as subunit interactions, and the regulation of the interaction by proteolytic modification of factor VIII and/or vWF.

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