Sedimentation Analysis of von Willebrand and Factor VIIIc Protein Using Partition Cells in the Analytical Ultracentrifuge

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

Sedimentation analysis of factor VIII complex was performed in the analytical ultracentrifuge using partition cells. This method allowed for the calculation of three different sedimentation coefficients from each run: one based on ristocetin agglutination activity for von Willebrand protein, $S_{rv}$; one based on coagulant activity for factor VIIIc, $S_{vIIIc}$; and one based on the schlieren or adsorption data for protein concentration, $S_{conc}$. In most cases, there was no agreement between the three values calculated from the same run, indicating a heterogeneous system. The calculated functional sedimentation coefficients give values that require the molecules to be highly asymmetric to be consistent with a glycoprotein of high molecular weight, which is in agreement with results observed in electron microscope studies. The dissociation of VIIIc into a smaller form can be demonstrated by this method. Determination of the three sedimentation coefficients in a series of fractions from gel filtration indicates a uniform size for the VIIIc activity but not for the WF activity. These observations are in agreement with the concept of a copolymer between WF and VIIIc and also with the concept of separate polymers for the two activities.

THE HUMAN FACTOR VIII–von Willebrand protein complex possesses two major biologic properties: the factor VIIIc (FVIIIc) coagulant activity, which will correct the long clotting time of patients with hemophilia A, and the von Willebrand activity, which is critical in supporting normal platelet–surface and platelet–platelet interactions that are lacking in patients with von Willebrand disease.1,4 Various genetic, biologic, as well as immunochemical, observations have led to the current concept that this complex circulates as a noncovalent association of two different proteins, one containing the factor VIIIc (FVIIIc) and the other responsible for the von Willebrand factor activity.3,9 Characterization of the “purified” von Willebrand factor has shown it to consist of multimers of a glycoprotein of molecular weight greater than $10^6$.10-13 However, studies of the protein in plasma have reported a smaller size.8,19 Although there is general agreement that the molecular weight of the FVIIIc protein is considerably smaller, the reported molecular weight values have ranged from 25,000 to 310,000.5,20,24

The present article examines the sedimentation behavior of the factor VIII complex using partition cells in the analytical ultracentrifuge.24-28 This method allows for the calculation of sedimentation coefficients ($S_{20,w}$) using functional activities as well as protein concentration. Results show that the von Willebrand protein behaves as a heterogeneous population of molecules, whereas the FVIIIc appears to be more homogeneous. The FVIIIc activity, when separated from the von Willebrand protein, sediments in accordance to previous reports23,27 and, when complexed, appears to be associated with only a unique family of von Willebrand multimers or is independently polymerized.

MATERIALS AND METHODS

Purification of Human von Willebrand Protein

Cryoprecipitate was provided by the American Red Cross, Rochester Regional Blood Program (Dr. J. Nusbacker, Director). After washing with ethanol and absorption with aluminum hydroxide and bentonite, the cryoprecipitate was processed by a two-step polyethylene glycol (PEG) 6,000 (Carbowax, Fisher Scientific, King of Prussia, PA) precipitation. During the first step, a 40% PEG-6,000 solution [0.02 M imidazole, 0.25 M sodium chloride, 0.02 M epsilon amino caproic acid (EACA), 200 U/mL aprotinin (Mobay Chemical Co., New York, NY), pH 6.5] was added to a final concentration of 2% and incubated for 30 min at 20°C. In the second step, PEG-6,000 was added to the supernatant fluid to a final concentration of 10%, and after centrifugation, the precipitate obtained was dissolved in a minimal amount of $\beta$-alanine buffer (0.3 M $\beta$-alanine, 0.15 M sodium chloride, 0.01 M sodium phosphate, 0.02 M EACA, 10 U aprotinin/mL, pH 6.8). Protein concentration was measured with folin-phenol reagent.28 Aliquots of the protein, dissolved in 2%-10% PEG precipitate (2%-10% PEG cut), were used directly in the sedimentation analysis experiments or were applied to a $130 \times 8.5$ cm Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ) column, as previously described.17 Following chromatography, the fractions emerging at elution volumes between 3 and 4 liters were pooled and concentrated by dialysis against 20% PEG-20,000 in $\beta$-alanine buffer and then used for the sedimentation studies. These purified concentrated fractions of von Willebrand protein contained less than 10% IgM, fibrinogen, $\alpha_2$-macroglobulin, fibroneciten, and $\beta$-lipoprotein by immunologic testing.

Human Endothelial Cell Conditioned Culture Medium

The human endothelial cell conditioned cultured medium was a gift from Dr. F. M. Booyse of the Michael Reese Research Foundation, Chicago, IL, and is identical to that described by him.29
Sedimentation

All runs were performed in a Model E Analytical Ultracentrifuge equipped with scanner optics. The runs were made using either the fixed partition cell\textsuperscript{12} or the moving partition cell.\textsuperscript{28} The technique used with the partition cells is that suggested by Schachman\textsuperscript{29} and includes the integration of the time necessary for the rotor to reach speed and to stop in the assessment of the total time for sedimentation ($\omega t$). Runs were made at or near room temperature using speeds ranging from 30,000 to 48,000 rpm. Normal sedimentation coefficients, employing schlieren optics (refractive index) or scanner optics (optical density) were calculated using the maximum ordinate for the schlieren pattern and the half wave height for the absorption system.

Functional sedimentation coefficients were calculated using the equation:

$$S = -\frac{1}{2} \frac{\ln \left( \frac{x_m^2 - x_p^2}{x_p^2} \right)}{\omega t}$$

where $\omega$ = angular rotation in radians/sec; $t$ = time in sec; $x_m$ = radial position of the meniscus; $x_p$ = radial position of the partition; $C_p$ = concentration of thoroughly mixed solution from upper compartment; and $C_m$ = concentration of the starting solution.

The three different sedimentation coefficients are designated $S_{conc}$ for optical data, $S_{VIIIc}$ for coagulant activity, and $S_{VIIIC}$ for ristocetin activity. All three assays were corrected to standard conditions of 20°C and water ($S_{0,0}$) by standard methods.\textsuperscript{30}

Functional assays

Ristocetin cofactor activity was measured by adding 50 $\mu$l of test sample to a mixture of 20 $\mu$l of ristocetin sulfate (50 mg/ml, Lenau, Copenhagen V, Denmark) and 0.8 ml of formalin-fixed human platelets.\textsuperscript{25} Activity was determined from the initial rate of platelet agglutination as measured by the change in optical density using a dual-channel platelet aggregometer at 37°C and 1,400 rpm stirring speed (Model S.H. 2336-F, Payton Associates, Buffalo, NY).

The results are plotted arithmetically as a function of dilution of the test sample; the value of $C_p/C_m$ from equation 1 is calculated as the ratio of slopes for the top compartment to the starting solution. This ratio is linear over the concentration range used in the experiments.

Factor VIII coagulant activity was determined by a modified activated partial thromboplastin time test using citrated plasma from a patient with severe hemophilia as the source of factor VIII-deficient plasma.\textsuperscript{25} The degree to which the abnormal partial thromboplastin time was corrected reflects the factor VIII activity of the test sample.

The results are plotted on a semilog plot of clotting time versus dilution. The value of $C_p/C_m$ from equation 1 is calculated from the parallel line response.

RESULTS

To ascertain the best operating conditions, runs were performed as a function of speed from 30,000 rpm to 48,000 rpm and time variations from 15 to 60 min. The value for $C_p/C_m$ varied from 0.90 to 0.08, but the calculated sedimentation coefficients were constant within experimental error. The biggest variations were at the lower speeds, where small quantities of material were transported, and at high speed for long times when very large portions of the material was transported and the amount remaining approached the limits of the assay. The best overall results were at 48,000 rpm for 30 min, and these conditions were used routinely except where noted.

To evaluate the reproducibility of the method at 48,000 rpm and 30 min, 8 identical runs were made on the PEG fraction and each top analyzed separately. The value for $S_{VIIIC}$ was 14.0 ± 0.6 and for $S_{VIIIc}$ 18.0 ± 0.5; this difference is statistically significant ($p < 0.05$). The values for $C_p/C_m$ ranged from 0.176 to 0.290, depending on the assay used and the position of the meniscus. The material remaining from the 8 top compartments was pooled and rerun at 40,000 rpm for 25 min to assure ample material in the top compartment, and the assays were 14.7 and 17.4 for $S_{VIIIC}$ and $S_{VIIIc}$, respectively.

Partition cell sedimentation runs were then made on a series of samples ranging from blood plasma directly to pooled purified activity from a Sepharose column; the three different sedimentation coefficients were calculated and are shown in Table 1. As can be seen, the value of the sedimentation coefficient for $S_{VIIIc}$ was that of the order of 19S, while the $S_{VIIIC}$ appeared to be smaller; none of the $S_{conc}$ values appear to correspond to the functional values.

The influence of high salt on the functional sedimentation coefficients is shown in Table 2. The sample was a PEG fraction that was analyzed initially at 48,000 rpm for 30 min. To a duplicate sample, solid NaCl was added to bring the salt concentration to 1 $M$, and the sample was rerun under identical conditions. The results show a reduction in the $S_{VIIIc}$ value from 18.9 to a value of 7.4, whereas the $S_{VIIIC}$ remained approximately the same at 20.4 and 19.3, respectively.

Table 1. Functional Sedimentation Coefficients on Different Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>$S_{VIIIC}$</th>
<th>$S_{VIIIc}$</th>
<th>$S_{conc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>20.4</td>
<td>16.4</td>
<td>—</td>
</tr>
<tr>
<td>Cryo ppt</td>
<td>19.7</td>
<td>15.9</td>
<td>24.3, 11.0, 6.8, 4.4</td>
</tr>
<tr>
<td>Cryo supernatant</td>
<td>18.2</td>
<td>17.1</td>
<td>—</td>
</tr>
<tr>
<td>PEG, 2%–10% fractions</td>
<td>18.4</td>
<td>14.9</td>
<td>12.7, 10.9, 5.9</td>
</tr>
<tr>
<td>Sepharose CL-2B (pool)</td>
<td>19.7</td>
<td>17.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Endothelial cell conditioned media</td>
<td>—</td>
<td>11.8</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 2. Effect of 1 $M$ NaCl on Functional Sedimentation Coefficients

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$S_{VIIIC}$</th>
<th>$S_{VIIIc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>18.9</td>
<td>20.4</td>
</tr>
<tr>
<td>Buffer and 1 $M$ NaCl</td>
<td>7.4</td>
<td>19.3</td>
</tr>
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</table>
remain constant, within experimental error, at approximately 18S.

To ascertain if the results obtained from the samples originating from plasma were dependent on purification methods, a sample of conditioned media from a culture of human endothelial cells was analyzed directly without further treatment. The only functional assay possible is for von Willebrand protein, and the calculated value for $S_{wW}$ was found to be 11.4 and 12.0 on two samples.

### DISCUSSION

It has long been suggested that a necessary criterion for homogeneity of a protein should be the calculation of an identical sedimentation coefficient when measured by schlieren pattern (refractive index) and by functional activity in a partition cell. It would appear that the WF protein approaches this condition at the point of its elution from the Sepharose CL-2B column (Table 3).

It has been suggested that the high molecular weights observed by some investigators resulted during the purification steps from blood plasma. These data do not support that concept, as the value from plasma to the purified material from gel filtration columns shows no large increase in sedimentation values. It is of interest that the sedimentation value obtained from the cryoprecipitate and the cryosupernatant are similar, even though greater than 90% of the activity partitions into the precipitate.

If we accept our previously reported molecular weights from SDS gels and combine that information with the functional sedimentation coefficients from this research, one can calculate the resultant Stokes radius ($R_s$) using an equation derived from Mann et al. and a frictional ratio, $f/f_r$, using the formula derived by Svedberg. These calculations for the fractions from Sepharose CL-2B are shown in Table 4. The possibility that the asymmetry of the molecule is indeed the correct situation is supported by the electron microscopic data on WF reported by Ohmori et al. and by our data and that of others, indicating that this material is almost excluded from Sepharose CL-2B, with an exclusion limit approaching a molecular weight of $40 \times 10^6$.

Our data on FVIIIIC obtained in high salt confirms the data of others that the FVIIIIC activity can be dissociated from a larger molecule. Our value of $S_{VIII}$ of 7.4 is in reasonable agreement with the value of 8.2 obtained by Hoyer and Trabold using sucrose density gradients. Using their value for the Stokes radius of 88 Å, we calculate a molecular weight of 250,000, using a partial specific volume of 0.70 as a "reasonable" value for a glycoprotein.

While the $S_{VIII}$ shows a marked decrease in high salt, the $S_{wW}$ remains virtually unchanged. One would normally expect a concomitant decrease in size of the WF moiety following the dissociation, unless the number of WF molecules is far greater than the number of VIIIIC molecules. This possibility seems likely and is supported by the observation that the $S_{visc}$ mimics the $S_{wW}$ much more closely than the $S_{VIII}$. This observation, along with the observation that the $S_{VIII}$ is constant throughout the gel filtration pools (Table 3), leads one to some structure possibilities. Our data indicate that the WF activity is homogeneous, consisting of an indefinite series of polymers. The sedimentation data correlate with the gel filtration elution data in that the S values decrease progressively as the elution proceeds. This is in contrast to the VIIIIC activity, which requires a combination of VIIIC with a discrete form of WF to explain the constancy of the

### Table 3. Functional Sedimentation Coefficients on Sepharose CL-2B Fractions

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>$S_{visc}$</th>
<th>$S_{vis}$</th>
<th>$S_{wW}$</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>17.4</td>
<td>23.0</td>
<td>23.0</td>
</tr>
<tr>
<td>2</td>
<td>16.8</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16.9</td>
<td>20.5</td>
<td>20.5</td>
</tr>
<tr>
<td>4</td>
<td>16.9</td>
<td>20.7</td>
<td>18.7</td>
</tr>
<tr>
<td>5</td>
<td>16.8</td>
<td>18.1</td>
<td>16.9</td>
</tr>
<tr>
<td>6</td>
<td>17.8</td>
<td>16.7</td>
<td>12.7</td>
</tr>
<tr>
<td>7</td>
<td>16.7</td>
<td>15.2</td>
<td>12.4</td>
</tr>
<tr>
<td>8</td>
<td>17.8</td>
<td>13.0</td>
<td>11.2</td>
</tr>
</tbody>
</table>

### Table 4. Calculated Values for Stokes Radius and Frictional Ratios

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Molecular Weight* ($\times 10^4$)</th>
<th>$S_{vis}$ ($\times 10^{15}$)</th>
<th>Stokes Radius† (Å)</th>
<th>Frictional Ratio ‡ ($f/f_r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10.0</td>
<td>1,532</td>
<td>11.3</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>21.3</td>
<td>1,488</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>20.5</td>
<td>1,375</td>
<td>11.0</td>
</tr>
<tr>
<td>4</td>
<td>6.4</td>
<td>20.7</td>
<td>1,089</td>
<td>9.4</td>
</tr>
<tr>
<td>5</td>
<td>5.4</td>
<td>18.1</td>
<td>1,051</td>
<td>9.6</td>
</tr>
<tr>
<td>6</td>
<td>5.4</td>
<td>16.7</td>
<td>1,139</td>
<td>10.4</td>
</tr>
<tr>
<td>7</td>
<td>4.6</td>
<td>15.2</td>
<td>1,066</td>
<td>10.3</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>13.0</td>
<td>813</td>
<td>9.0</td>
</tr>
</tbody>
</table>

*Taken from SDS gel electrophoresis data of Martin.
†Calculated from molecular weight and S by equation derived by Mann.
‡Calculated from molecular weight and S by equation derived by Svedberg.
size. This observation then puts some important constraints on the possibility that a hybrid molecule exists. It is therefore quite plausible, based on these results, that each of these activities reside in a unique polymer. The VIIIC activity residing in a molecule with a size corresponding to 18S, which can dissociate into 3 or 4 subunits of 7.5S, while the WF activity resides in a molecule that forms an indefinite series of polymers, with the smallest in these experiments corresponding to an S of 12–14. The data on the endothelial cell conditioned media may tend to support the separate polymer concept, as, for WF, the sedimentation value is consistent with the value of S = 12–14 and there is no VIIIC activity present.

REFERENCES

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