von Willebrand factor (VWF)2 is a large, multidomain glycoprotein that is present in human blood and in secretory granules of endothelial cells and platelets (1–3). This protein occurs both as a protomer and in multimeric form. The ~500-kDa protomer consists of two identical monomer subunits linked at the C terminus by disulfide bonds. Linear multimers formed by cysteine-cysteine linkages near the N terminus result in a molecular mass of >10,000 kDa.

VWF serves many functions. The binding of surface-immobilized VWF to platelet receptor GpIbα results in intermolecular bonds with high tensile strength (4, 5). This molecular interaction allows platelet capture at sites of vascular injury under high fluid shear conditions. The binding of plasma VWF to platelet receptor GpIbα under high hydrodynamic shear also leads to platelet activation and subsequent platelet arrest (6). Various mutations in VWF result in the bleeding defects that characterize von Willebrand disease (1). In blood, VWF binding to pro-coagulation factor VIII increases factor VIII lifetime in circulation. Finally, the size of VWF and its response to fluid flow are key determinants in regulating protein function under physiological and pathological conditions. In support of this, the life threatening systemic illness thrombotic thrombocytopenic purpura (TTP) is attributed to the presence of very large VWF multimers, which are caused by the malfunction or absence of a metalloprotease termed ADAMTS-13 (“a disintegrin and metalloprotease with thrombospondin” family member) (7). VWF cleavage by ADAMTS-13 is enhanced both upon protein denaturation with guanidine-HCl (Gd-HCl) (8) and upon application of physiological fluid shear (9).

Structural studies on VWF have been performed using two-dimensional electron microscopy (10–12). These studies show that multimeric VWF consists of a repeating protomer unit that has a maximum extended length of 120 nm (12) (Fig. 1). This protomer has 2-fold symmetry, with each half consisting of a long flexible 34 × 2-nm rod linked to a large globular 22 × 6.5-nm domain at one end and to a second rod at the other end. Using rotary-shadowed electron microscopy of VWF deposited on mica surfaces, it has been shown that whereas 87% of multimeric VWF adopt a “ball of yarn” or “tangled” conformation with a mean diameter of 100–150 nm, the remaining exist in an “extended form” with a mean length of 350 nm (10). Atomic force microscopy (AFM) measurements of VWF immobilized on different substrates further suggest that the VWF structure determined using microscopy depends on the surface on which it is deposited. On hydrophobic octadecyltrichlorosilane-modified glass, VWF exhibits a compact structure, whereas the molecule is more extended on hydrophilic mica (13).

The detailed solution structure of VWF is yet to be determined. Whereas light scattering provides good estimates of hydrodynamic radius ($R_h$) and radius of gyration ($R_g$), this method does not provide detailed information on molecular...
amino homobifunctional cross-linker BS3 (bis[sulfosuccinimidy]suberate) also reveal, for the first time, that VWF solution structure is stabilized by frequent interdomain interactions between residues located on different monomer units. Such interdomain interactions may be important regulators of VWF function in normal physiology and pathology.

**EXPERIMENTAL PROCEDURES**

**Materials**—Polyclonal rabbit antihuman VWF was from Dako (Carpinteria, CA). Non-inhibitory anti-VWF mAb AVW-1 and A1 domain function blocking mAb AVW-3 were from GTI Diagnostics (Waukesha, WI). Polyclonal rat antifactor VIII antibody and recombinant factor VIII were kindly provided by Dr. Balasubramanian (SUNY-Buffalo). F(ab′)2, Alexa 488-conjugated secondary antibodies were from Invitrogen. All chemicals were from Sigma unless otherwise specified.

Denaturant Gd/HCl was deuterated by dissolving it in 99.9\% D2O (Cambridge Isotope, Andover, MA) at 4 M and subjecting the solution to three cycles of evaporation using a vacuum oven, with resuspension in D2O at the end of each cycle (20). The final product, which is abbreviated Gd/DCl, was completely deuterated as determined by proton NMR spectroscopy (Varian Inova-500, Fort Collins, CO) and solid phase fourier transform infrared spectroscopy (Mattson Galaxy series FT-IR 5000, Madison, WI).

Multimeric human VWF was purified from blood plasma cryoprecipitate obtained from Community Blood Bank (Erie, PA) (14). Silver staining of SDS-PAGE gels confirmed that a unique protein was isolated.

**VWF Protomer (ΔPro-VWF) Expression**—Full-length VWF contains 2813 amino acids including a 741-amino acid propeptide. Deletion of this propeptide results in dimeric ΔPro-VWF formation with protomer units linked by disulfide bonds at the C, but not N terminus (21). To produce ΔPro-VWF for small angle scattering studies, cDNA was purchased from ATCC (Manassas, VA) in the PMT-2-ADA vector. This construct was digested with EcoRI, and full-length VWF was cloned into pcDNA3.1(+)/Myc-His (Invitrogen, Carlsbad, CA) (VWF-pcDNA). VWF-pcDNA was then digested using BamHI, and the excised fragment of VWF was ligated into pBluescript sk(+)(Stratagene). The propeptide portion of VWF (residues Ala23–Arg763) was deleted in this vector by performing a PCR with 5′-phosphate sense 5′-AGCCTATCCGTGCgGCCtCCaATGGTCAAGCTG-3′ and antisense 5′-ACAAAAGGTtCtCtGACAAATGAG-3′ primers. During this PCR step, silent mutations at C2307T and C2310A were introduced to facilitate primer design. The PCR product thus formed was purified and blunt end-ligated (del-BamHI-VWF-Blue). del-BamHI-VWF-Blue was digested with BamHI, and the VWF fragment was ligated back into the BamHI-digested VWF in pcDNA (ΔPro-VWF-pcDNA). Restriction enzyme digests were performed to verify correct orientation of insert, and the final product was sequenced.

CHO-S cells (Invitrogen) were transfected with ΔPro-VWF-pcDNA using FuGENE 6 (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer’s instructions. Stable clones were obtained by selecting with 2 mg/ml G418 (Invitrogen). After selection, the antibiotic concentration was reduced to 0.5 mg/ml. Unique colonies were obtained by limiting dilution and were then scaled up for protein purification. The cultures were converted to protein-free CD-CHO media (Invitrogen), and the cell culture supernatant was harvested for ΔPro-VWF purification.

A two-step procedure was used to purify ΔPro-VWF from CD-CHO culture medium. In the first step, anion exchange chromatography was applied using Fractogel® EMD TMAE (M) (EMD Chemicals, Gibbstown, NJ) and FPLC system from Amersham Biosciences/GE Healthcare Biosciences (Piscataway, NJ) (22). This column was first equilibrated with 20 mM Tris buffer, pH 7.4. Cell culture supernatant containing ΔPro-VWF was diluted 1:1 in 20 mM Tris buffer (final pH 7.2), and this was run through the column twice. VWF binding to column was verified by assaying negligible VWF concentration in culture supernatant following passage through ion-exchange column, using the flow cytometry-based VWF concentration assay described below. Following binding, VWF was eluted using 20 mM Tris buffer by increasing NaCl concentration in a stepwise manner from 150 and 260 mM up to 400 mM. VWF was observed to elute when 260 mM NaCl was applied. Fractions containing ΔPro-VWF were thus pooled. In the second step, Microsep™ 100-kDa cutoff centrifugal devices (Pall Life Sciences, Ann Arbor, MI) were used to remove low molecular weight impurity. First a 15-fold concentration of the pooled fractions was performed. Following this, two more sequential cycles of centrifugal concentration were performed with ΔPro-VWF, with protein volume increased by 10-fold using 20 mM Tris buffer before each centrifugation step, and 10-fold concen-
tration was accomplished. Fresh Microsep units were used for each centrifugal concentration step. Following the third and final centrifugal step, theoretically, >99% of the low molecular impurities should have been removed.

Protein Concentration Determination—VWF concentration was measured using three methods. In the case of purified protein where concentration was high (>1–2 μg/ml), the Coomassie/Bradford protein assay kit (Pierce) was employed.

In complex protein mixtures and at lower concentrations down to 50 ng/ml, VWF concentration was assayed using a flow cytometry assay. For this, mAb AVW-1 was covalently immobilized on 5.7-μm polystyrene microbeads (Poly-sciences, Warrington, PA) using carbodiimide chemistry (23). These beads are called anti-VWF beads. Polyclonal anti-VWF mAb (Dako) was conjugated with FITC. Multimeric VWF protein standards were also developed for this assay based on measurement of protein concentration using the Coomassie protein assay kit. During each run, VWF (unknown samples or standards at varying doses) was incubated with 10⁶ anti-VWF beads/ml for 20 min at room temperature, washed, and then 1:20 (v/v) FITC-conjugated polyclonal anti-VWF was added to these beads. Following 20 min, the sample was again washed. FITC fluorescence associated with the beads was then measured by FACS Calibur flow cytometry (BD Biosciences, San Jose, CA) to quantify VWF concentration. As shown in supplemental Fig. S1, this assay detected protein concentration down to ∼50 ng/ml, and sample readouts were linear over at least two logs (0.1–10 μg/ml).

Quantitative amino acid analysis was further performed at the Protein Chemistry Laboratory (Texas A&M University), and results from this absolute measurement were used to calibrate both the Bradford and flow cytometry assays described above. Thus, the VWF concentrations presented in this article are based on amino acid analysis. Here, we observed that the Bradford assay overpredicts VWF concentration by ∼6%. The concentration of individual amino acids assayed using amino acid analysis was also within 10% of that predicted based on protein primary sequence. This is reasonable for a purified protein.

Electrophoresis—Western blotting of VWF was performed using 0.6% agarose gel (14). Silver staining of gradient (4–20%) SDS-PAGE gels was performed using Silver SNAP stain Kit II (Pierce). Densitometry analysis was performed using the Kodak 1-D software (New Haven, CT).

In studies with BS3, the homobifunctional cross-linker was immobilized on 5.7-μm polystyrene microbeads (Poly-sciences, Warrington, PA) using carbodiimide chemistry (23). These beads are called anti-VWF beads. Polyclonal anti-VWF mAb (Dako) was conjugated with FITC. Multimeric VWF protein standards were also developed for this assay based on measurement of protein concentration using the Coomassie protein assay kit. During each run, VWF (unknown samples or standards at varying doses) was incubated with 10⁶ anti-VWF beads/ml for 20 min at room temperature, washed, and then 1:20 (v/v) FITC-conjugated polyclonal anti-VWF was added to these beads. Following 20 min, the sample was again washed. FITC fluorescence associated with the beads was then measured by FACS Calibur flow cytometry (BD Biosciences, San Jose, CA) to quantify VWF concentration. As shown in supplemental Fig. S1, this assay detected protein concentration down to ∼50 ng/ml, and sample readouts were linear over at least two logs (0.1–10 μg/ml).

Quantitative amino acid analysis was further performed at the Protein Chemistry Laboratory (Texas A&M University), and results from this absolute measurement were used to calibrate both the Bradford and flow cytometry assays described above. Thus, the VWF concentrations presented in this article are based on amino acid analysis. Here, we observed that the Bradford assay overpredicts VWF concentration by ∼6%. The concentration of individual amino acids assayed using amino acid analysis was also within 10% of that predicted based on protein primary sequence. This is reasonable for a purified protein.

Electrophoresis—Western blotting of VWF was performed using 0.6% agarose gel (14). Silver staining of gradient (4–20%) SDS-PAGE gels was performed using Silver SNAP stain Kit II (Pierce). Densitometry analysis was performed using the Kodak 1-D software (New Haven, CT).

In studies with BS3, the homobifunctional cross-linker was immobilized on 5.7-μm polystyrene microbeads (Poly-sciences, Warrington, PA) using carbodiimide chemistry (23). These beads are called anti-VWF beads. Polyclonal anti-VWF mAb (Dako) was conjugated with FITC. Multimeric VWF protein standards were also developed for this assay based on measurement of protein concentration using the Coomassie protein assay kit. During each run, VWF (unknown samples or standards at varying doses) was incubated with 10⁶ anti-VWF beads/ml for 20 min at room temperature, washed, and then 1:20 (v/v) FITC-conjugated polyclonal anti-VWF was added to these beads. Following 20 min, the sample was again washed. FITC fluorescence associated with the beads was then measured by FACS Calibur flow cytometry (BD Biosciences, San Jose, CA) to quantify VWF concentration. As shown in supplemental Fig. S1, this assay detected protein concentration down to ∼50 ng/ml, and sample readouts were linear over at least two logs (0.1–10 μg/ml).

Quantitative amino acid analysis was further performed at the Protein Chemistry Laboratory (Texas A&M University), and results from this absolute measurement were used to calibrate both the Bradford and flow cytometry assays described above. Thus, the VWF concentrations presented in this article are based on amino acid analysis. Here, we observed that the Bradford assay overpredicts VWF concentration by ∼6%. The concentration of individual amino acids assayed using amino acid analysis was also within 10% of that predicted based on protein primary sequence. This is reasonable for a purified protein.
99.9% D₂O. The goal of this dialysis was to remove residual hydrogenated material that can contribute to the incoherent scattering of neutrons. Detailed methods including instrument settings and data analysis protocols are provided under supplemental materials. A brief overview follows.

SANS measurements were performed on the 30-m NG3 SANS spectrometer at the NIST Center for Neutron Research (Gaithersburg, MD) (24). During experimentation, 0.094–0.28 mg/ml multimeric or 0.15 mg/ml protomer VWF was placed in the path of a 6-Å neutron beam at room temperature. In some runs, VWF was incubated with 1.25 mM Gd(DCl)₃ for 4–5 h before SANS measurement. SANS experiments produce data that quantify scattering intensity, I(q), as a function of the scattering vector, q. In general, q is a measure of instrument magnification (25), and it is defined as 4π sin(θ/2), with θ representing the scattering angle and λ the neutron wavelength. Because scattering angle (θ) is related to the macromolecule length scale under study, the I(q) versus q plot contains information on protein solution structure. The q range applied in our experiments allows study of proteins in the size range from 2–250 nm.

SANS data were analyzed using three complementary approaches. First, VWF Rg was estimated by plotting 1/I(q) versus q² and fitting data using the Cattell approximations (26). Only a few points at the lowest q range were used for this analysis because this analysis method is only valid when qRg < 4. The intercept of this plot yields 1/I(0), and Rg is obtained using Rg = (3 × slope/(0.359 × intercept))¹/². Second, the overall protein shape was determined by fitting the I(q) versus q data in the low and moderate q range, with the model of an ellipsoid using software available from NIST. Output parameters from this fit were the major and minor axis radii, rₐ and rₜ. The radius of gyration Rg was calculated using Rg = rₐ((rₐ² + 2rₜ²)/5)⁰.⁵, and this was within 20% of the value obtained from Calmettes analysis. Finally, higher resolution fitting of data over the entire q range was performed using the unified equation (27). According to this, scatter arising from a complex morphology over a wide q range is the result of individual contributions from substructural elements that span a smaller q range. In this context, each structural level resembles a plateau at low q in the I(q) versus q plot followed by a power law decay at higher q (27). The scattering intensity at the low q plateau is defined by the parameter Gᵣ (Guinier prefactor), the point where the decay begins is defined by Rgᵣ (Rg of the iᵗʰ structural level) and Pᵣ (power law exponent) defines the decay-slope at high q. Gᵣ is thus a measure of neutron intensity at the start of the iᵗʰ structural feature, and it equals I(0) in the case of the first structural level. Depending on protein concentration and extent of VWF multimer formation, Gᵣ for lower structural levels can differ between different VWF preparations. Pᵣ contains information about the features of the substructure: 4 > Pᵣ > 3 for surface fractals, Pᵣ < 3 for mass fractals, and Pᵣ > 4 for diffuse interfaces. Radii of consecutive structural levels (Rgᵣ and Rgᵣ₊₁) define the bounds of each structural level. Using a piecewise fitting algorithm described under supplemental materials, Gᵣ, Rgᵣ, and Pᵣ were determined for substructures that form multimeric and protomer VWF.

Dynamic Light Scattering—Dynamic light scattering experiments were performed using a Brookhaven Goniometer (BI-200SM Ver.2.0, Brookhaven Instruments, Holtsville, NY) with a 514-nm laser and autocorrelator (model:BI-9000AT) (14). These experiments yielded VWF hydrodynamic diameter distribution function on a volume basis.

Statistics—All data are presented as mean ± S.E., except for SANS data where error bars represent S.D.

RESULTS
Light and neutron scattering studies were performed with protomer and multimeric VWF. Efforts were undertaken to characterize the protein function so that structural results could be attributed to functional macromolecules.

Functional Characterization of Protomer VWF (ΔPro-VWF)—For studies with ΔPro-VWF, this protein was expressed in stably transfected mammalian CHO cells. After scale up, the flow cytometry-based protein assay demonstrated that the cell cul-
tature supernatant of transfected CHO cells yielded ΔPro-VWF at ~1.88 μg/ml. This was purified and concentrated to 150 μg/ml. Fig. 2A compares the Western blots for multimeric and ΔPro-VWF. As seen, ΔPro-VWF has a molecular mass of ~500 kDa, which corresponds to the lowest mass band of the multimeric protein. Mutimeric VWF preparations had a size up to 20–30 mers as determined using densitometry. Silver staining and densitometry analysis of reduced ΔPro-VWF (~250 kDa in Fig. 2B) demonstrated this protein was ~93% pure. Lower density bands of 45 and 37 kDa were also present. The lower mass bands remain even after repeated centrifugal filtration, suggesting that these were non-covalently associated with VWF when the protein was secreted from CHO cells. Addition of DTT and SDS prior to electrophoresis likely resulted in their dissociation from ΔPro-VWF. There is a minimal contribution of lower MW proteins to ΔPro-VWF scanning data as discussed later.

ΔPro-VWF was functional in terms of its ability to bind factor VIII (Fig. 2C). In this sandwich-cytometry assay, which independently measured the binding of VWF to anti-VWF beads and of factor VIII to VWF immobilized on these beads, we determined that multimeric VWF from plasma cryoprecipitate had low levels of factor VIII bound to it. This is consistent with dissociation constant (K_D = 0.4 nM) and off-rate (k_off = 2.3 x 10^−4 s) measurements for factor VIII-VWF interaction (28). Based on these binding affinities, it is expected that whereas factor VIII is bound to VWF during protein isolation from blood, it may have dissociated over a period of days prior to the experiments described here. Indeed, factor VIII binding to multimeric VWF could be readily restored upon addition of recombinant exogenous factor VIII. The multimeric protein bound factor VIII more efficiently than dimeric ΔPro-VWF, suggesting that the multimeric nature of VWF may enhance binding interactions.

The function of the A1-domain of ΔPro-VWF was confirmed using a platelet-VWF bead adhesion assay under shear (Fig. 2D). Here, platelets were observed to readily engage VWF-bearing beads when fluid shear rate was applied at 1785/s. Specificity was confirmed using blocking antibody against VWF A1-domain.

ΔPro-VWF Has an Rg of 30 nm and 4.5-nm Subdomains—Light scattering and SANS studies were performed with purified dimeric ΔPro-VWF to characterize its solution structure. Dynamic light scattering showed that protein hydrodynamic diameter D_h varied from 15–50 nm (Fig. 3A). Similar values of D_h were obtained when protein concentration was varied from 37 to 150 μg/ml, suggesting that these measurements are reliable. The relatively narrow distribution of D_h values suggests that ΔPro-VWF may not aggregate spontaneously in solution.

SANS analysis of ΔPro-VWF was performed. Calmettes analysis (26) of SANS data at q < 0.09/nm (Fig. 3B) yields an I(0) value of 0.19/cm and R_g of 32.6 nm. Using these estimates as a starting point, SANS data over the entire q range were fit to the model of a monodispersed ellipsoid (Fig. 3C). A prolate ellipsoid (shape of an elongated rugby ball) with R_p, r_p, and r_a values of 31.8, 70.0, and 9.1 nm, respectively, fit the data well at q < 0.2/nm. These parameter values are in reasonable agreement with previous electron microscopy measurements of protomer units (12). Because SANS data contain information over different length scales, the unified equation was next used to fit data over the entire q range (Fig. 3D). Two different structural levels were observed. The first level, which represents the overall structure of ΔPro-VWF, has G_1, R_g, and P_1 values of 0.3/cm, 30 nm, and 2.4, respectively. The smaller structural level has G_2, R_g, and P_2 values of 0.025/cm, 4.5 nm, and 3.0. This substructure may represent individual protein domains because the A1 and A3 domains of VWF are known to exist in the size range of 4 nm (16, 29). In addition, this substructure may also include information on the nature of interaction between protein domains. Because P_1 ~ 3 for both structural levels above, the structure of VWF has the features of a mass fractal (27). Upon considering ellipsoid dimensions of ΔPro-VWF and volume occupied by amino acids based on protein molecular composition (30), it is estimated that only ~2% of the ellipsoid is occupied by protein mass, whereas the remaining is water (supplemental materials). ΔPro-VWF is thus loosely packed compared with other proteins like BSA and fibrinogen (see “Discussion”).

Functional Characterization of Multimeric VWF—Studies were next performed to relate scattering data of individual pro-tomers to their arrangement in multimers. As part of this investigation, experiments were performed to confirm that the multimeric protein in our studies exhibited activity similar to plasma VWF. In the aPTT assay (Table 1), we determined the amount of factor VIII associated with VWF during protein isolation from plasma cryoprecipitate. These studies showed that a single factor VIII molecule was bound to ~30–200 VWF protomers in both purified VWF and blood plasma. These results are consistent with the published data (31). The isolated VWF
was also active in terms of its ability to bind platelet receptor GpIbα via its A1 domain and to activate platelets in a shear-induced platelet activation assay (see earlier publication, Ref. 14). In a final assay performed to confirm the function of purified multimeric protein, we determined that multimeric VWF did not undergo proteolysis by ADAMTS-13 unless this protein was denatured using Gd-HCl (supplemental Fig. S2). These studies confirm both the presence of functional VWF and the absence of denatured protein in our preparation.

Three Distinct Substructures Contribute to VWF Solution Conformation—Light and neutron scattering studies were undertaken with multimeric protein. Unlike the protomer, dynamic light scattering experiments showed that the hydrodynamic diameter of multimeric VWF ($D_g$) varied over a range from 130 to 200 nm (Fig. 4A). This is indicative of VWF polydispersity. Calmettes analysis of SANS data at $q < 0.04$ nm resulted in $I(0)$ values of 37.3 and $R_g$ of 97.3 nm (Fig. 4B). These estimates of $R_g$ are in agreement with previous results (14). Using the above $I(0)$ value, we observed that SANS data at $q < 0.1$ nm could be fit to a prolate ellipsoid with major and minor axis radius values of 175 and 28 nm (Fig. 4C).

Fig. 4D presents the unified equation fit. Three different structure levels were observed with multimers at $R_g \sim 75$ nm, intermediate structures at $R_g \sim 16$ nm, and domain level structural features at $R_g \sim 4.5$ nm. The 16-nm intermediate level likely represents the protomeric globule structure formed at the N terminus of two adjacent VWF monomers, as illustrated in the figure inset (12). Table 2 presents unified equation parameter values for 11 independent VWF samples analyzed using SANS. Some of these samples were from a single individual whereas others were pooled samples. Three structural levels were consistently observed in each of these samples with similar $R_g$, and $p_i$ values. This confirms that data presented in Fig. 4D are donor- and blood group-independent. The ratio of $G_2/G_3$ were constant at ten among the samples, because similar domain structures contribute to protomer structure in each protein. Further, some variation in the ratio of $G_1/G_2$ was noted (162.5 ± 19.4), and this reflects the different multimer distribution in each sample. Finally, the absolute value of $G_1$ varied because this was a function of protein concentration in addition to VWF size.

Guadinine-HCl Causes Local Changes in VWF Domain Structure without Altering Overall Shape or Size—We tested the ability of SANS to identify structural changes in VWF, because such analysis can aid future structure-function investigations. The deuterated denaturant Gd-DCl was used in these runs (Fig. 5), because treatment of VWF with this denaturant enhances VWF susceptibility to proteolytic cleavage by ADAMTS-13 (8). As seen, the $R_g$ of the multimer did not change upon denaturant treatment, whereas the slope of the scattering curve at the highest $q$ was reduced. This change in slope at length scales <10 nm supports the proposition that

### TABLE 1

Factor VIII content in purified multimeric VWF obtained from three donors

| Sample | Number of VWF protomers per factor VIII molecule/
|--------|---------------------------------------------------|
| Plasma | 36.5 ± 0.051                                      |
| B+ VWF | 221.9 ± 14.4                                      |
| O+ VWF | 51.4 ± 0.35                                       |
| A+ VWF | 46.1 ± 1.1                                        |

a Data are mean ± S.E.

### TABLE 2

Unified equation fit to SANS data for 11 different VWF preparations

| VWF sample number | $G_1$ /cm | $R_g$ /nm | $p_i$ | $G_2$ /cm | $R_{g,2}$ /nm | $P_2$ | $G_3$ /cm | $R_{g,3}$ /nm | $P_3$ |
|-------------------|-----------|-----------|-------|-----------|--------------|-------|-----------|--------------|-------|
| 1                 | 16        | 75        | 2     | 0.13      | 16           | 2.4   | 0.013     | 4.5          | 3     |
| 2                 | 8         | 75        | 2     | 0.04      | 15           | 2.4   | 0.004     | 4.5          | 3     |
| 3                 | 60        | 75        | 2     | 0.25      | 15           | 2.4   | 0.025     | 5.0          | 3     |
| 4                 | 13        | 75        | 2     | 0.05      | 15           | 2.6   | 0.005     | 4.5          | 3     |
| 5                 | 11        | 75        | 2     | 0.06      | 16           | 2.6   | 0.006     | 4.5          | 3     |
| 6                 | 50        | 75        | 2     | 0.4       | 16           | 2.4   | 0.04      | 4.5          | 3     |
| 7                 | 15        | 75        | 2     | 0.25      | 17           | 2.4   | 0.025     | 4.5          | 3     |
| 8                 | 20        | 75        | 2     | 0.22      | 17           | 2.4   | 0.022     | 4.5          | 3     |
| 9                 | 125       | 75        | 2     | 0.55      | 16           | 2.4   | 0.055     | 4.5          | 3     |
| 10                | 80        | 60        | 2     | 0.55      | 17           | 2.4   | 0.055     | 4.5          | 3     |
| 11                | 40        | 75        | 2     | 0.3       | 17           | 2.4   | 0.03      | 4.5          | 3     |
Gd·HCl alters domain level features, and this is sufficient for VWF proteolysis. These observations were also validated using dynamic light scattering where neither addition of 1.25 M Gd·DCl nor Gd·DCl altered protein $R_g$ (data not shown). In a control experiment (supplemental Fig. S3), we verified that 1.25 M Gd·DCl was as effective as 1.25 M Gd·HCl in increasing VWF susceptibility to proteolysis by ADAMTS-13. These experiments confirm that deuterated and hydrogenated forms of Gd·HCl are functionally similar.

**Non-covalent Interactions between Different Monomers of VWF Stabilize Protein Quaternary Structure**—The inability of Gd·HCl to alter $R_g$ and previous reports of VWF self-association (14, 32) suggest that there may be molecular interactions between various domains within a single VWF multimer. To assess this, VWF was randomly cross-linked with homobifunctional cross-linker BS³, which links proximal amine residues within the protein, prior to cleavage of intermonomer disulfide bonds using DTT and Western blot analysis (Fig. 6). As seen in A, cross-linking prior to reduction and blotting results in fragments with masses of 250, 500, and 750 kDa. In these studies, increasing the amount of cross-linker from 1 to 5 mM (lane 1 versus 2 and lane 4 versus 5) did not markedly affect the pattern of multimers formed. Similarly increasing the ratio of VWF to BS³ by altering VWF quantity (lane 1 versus 4, and 2 versus 5) did not significantly affect the blot results. Even treatment with cross-linker for a brief 2 min (lane 3) instead of 30 min (lane 1) resulted in similar results, and these data together suggest that BS³ cross-linking is a rapid process, and it likely stabilizes existing intramolecular binding interactions, rather than initiating new intermolecular interactions following Brownian collisions. In support of this, the negative control run, where VWF was treated with DTT prior to BS³ addition, only resulted in a single 250-kDa band.

Whereas the maximum size of cross-linked VWF was 750 kDa when a pooled sample of VWF consisting of both low and high molecular mass protein fractions was used (Fig. 6A), larger units were observed in similar studies performed with only the higher molecular mass fraction (Fig. 6B). In the later studies, cross-linked units consisting of at least six monomer units were observed. Thus, the extent of cross-linking depends on the protein multimer distribution. The above cross-linking studies were also performed in the presence of 0.1% SDS, which disrupts most self-associated VWF (16). In these runs also, we observed the ladder pattern of cross-linked VWF shown in Fig. 6. Finally BS³ treatment of dimeric ΔPro-VWF prior to blotting did not result in protein cross-linking as determined by Western blot analysis (data not shown). Because the intensity of cross-linked VWF (≥500 kDa) is typically greater than that of the monomer (250 kDa) in Fig. 6, the experiments suggest that interactions between the VWF domains located on different monomer units may be a “frequent” occurrence. Further, while the exact domains involved in the above interactions remains to be established, the occurrence of cross-linked units with >500 kDa (dimers) and the absence of cross-linking in runs with ΔPro-VWF is consistent with the notion that simple cross-linking of N terminus residues of VWF by BS³ alone does not account for the observations. Overall, the intraprotein interactions revealed by studies with BS³ may play a role in stabilizing VWF solution structure.

**DISCUSSION**

Small angle scattering experiments were performed to study the solution structure of VWF in the length scale from 2–250 nm. The results of this work complement previous crystallography studies of VWF, which have examined individual protein...
TABLE 3
Protein dimensions obtained from ellipsoid fit of SANS data

| Protein          | BSA | Fibrinogen | ΔPro-VWF | Multimer VWF |
|------------------|-----|------------|----------|--------------|
| $r_a$ (nm)       | 4.4 | 26         | 70       | 175          |
| $r_b$ (nm)       | 2.3 | 2.7        | 9.1      | 28           |
| Aspect ratio (= $r_a/r_b$) | 1.9 | 9.63       | 7.7      | 6.2          |
| $R_g$ (nm)       | 2.5 | 16.9       | 31.8     | 80.2         |
| Mass (kDa)       | 137.1 | 387.4     | 429.6    | - -          |
| Ellipsoid volume occupied by protein (% v/v) | 97.6 | 34.7      | 2.0      | 1.6          |

* Reliable mass values for multimeric VWF could not be obtained from SANS data because of its large size.

domains at smaller length scales (16, 29). Some of the gross structural features of VWF are presented in Table 3, where they are compared with our unpublished SANS results from two other proteins, BSA and human fibrinogen. Here, it is seen that all proteins can be modeled as prolate ellipsoids. This is in general agreement with the prediction of Kuhn (33) who suggested that the overall shape of a polymeric macromolecule undergoing random walk is aspherical. In the case of proteins, secondary and tertiary interactions between amino acid side chains likely cause the formation of ellipsoids with distinct aspect ratios. Thus protomer and multimer VWF have an aspect ratio of ~6–7. Fibrinogen is more elongated, and this is consistent with its crystal and electron microscopy structure (34, 35). BSA used in this study appears to be predominantly in the form of a dimer and appears more compact to the neutron beam compared with other proteins (36). The $I_D$ values from neutron scattering studies were used to estimate protein mass as discussed under supplemental materials, and these are in agreement with published data. The volume fraction of the ellipsoid occupied by both the protomer and multimeric forms of VWF was low (~1.6–2%) compared with the other proteins.

With regard to the substructural levels within VWF, three levels were consistently observed in the multimeric protein. Whereas VWF polydispersity affected the $G_1$ or $I_q$ values in the first structural level, the smaller substructures were similar and did not display any obvious differences among donors with different blood groups. These include the entire multimeric protein with $R_g$ of ~75 nm, the globular domain with $R_g$ of ~16 nm and mass fractal-like domain level structures with $R_g$ of 4.5 nm. Similar to this, two structural levels were observed in the protomeric VWF including the overall dimeric protein with $R_g$ of 30 nm and domains of 4.5 nm. The smaller domains noted above may correspond to individual functional units that have been previously crystallized (16). They may also contain information on the arrangement of individual domains in clusters. SANS data of comparable concentrations of multimeric and protomeric VWF are overlaid in supplemental Fig. S4, and these data match at $q > 0.3$ nm. Because lower mass (~40 kDa, Fig. 2B) contaminating proteins seen in SDS-PAGE runs are expected to affect scattering data at $q > 1.5$ nm (size range of 4 nm) and because the scattering of multimeric and protomer VWF is similar in this $q$ range, the results in supplemental Fig. S4 confirm that low mass molecules have only minor contribution to SANS experiments.

The structural features reported in this article likely represent those of native human VWF in blood plasma, because our macromolecules are functional. In this regard, both the recombinant and purified multimeric forms of VWF had the ability to bind platelet GpIb under fluid shear, and recombinant factor VIII in cytometry/clotting assays. Multimeric protein was also not denatured upon isolation, because the protease ADAMTS-13 did not cleave this protein unless denaturant Gd-HCl was added to alter protein conformation and expose sites of proteolysis.

This study presents evidence that individual domains on distinct VWF monomer units may interact in a non-covalent manner. In support of this, we observed that cross-linking VWF with BS3 results in the formation of stable amide bonds linking proximal amino acid residues on different monomer units. Previous studies also provide evidence of interactions between VWF units under static (15, 32) and fluid flow conditions (14, 37). In a study performed with 2-mercaptoethanol-reduced VWF (15), the authors report that protomer VWF may spontaneously self-associate in solution when they are at concentrations of >100 μg/ml, and that such binding is dissociated upon addition of 0.1% SDS. Based on our observation that ΔPro-VWF exhibits a sharp peak in dynamic light scattering studies over a range of solution concentrations, we suggest that the dimeric protein may not spontaneously aggregate. Thus, independent mechanisms may regulate the association of 2-mercaptoethanol-treated and non-denatured proteins. In other studies, non-denatured recombinant and plasma VWF in solution have been shown to bind surface immobilized VWF under fluid shear (37) and under static conditions (32). Further, VWF self-associates in suspension upon application of fluid shear (14). Although the detailed mechanisms of these interactions are not yet established, it is suggested that VWF self-association is specific in nature (32), and that it involves at least two different epitopes located on distinct portions of VWF. Further, these interactions do not involve the homotypic interaction between the A1 and A3 domains (37). Finally, VWF self-association can be partially dissociated by 0.1% SDS (14). In the current study, while addition of 0.1% SDS during the VWF cross-linking step did not prevent the action of the BS3 linker, it is noted that the conformation of VWF in these studies is also constrained by disulfide linkages between monomer units. Overall, more detailed investigations in the future may reveal whether similar or distinct mechanisms contribute to the different observations of VWF-VWF interaction described above. Further, it remains to be established to what degree the interactions between the domains contribute to protein ellipsoidal shape and function.

The exact protein interactions stabilized by BS3 in the cross-linking studies remains to be established, though our experiments suggest that these do not simply involve the linkage of proximal primary amines located near the N terminus of VWF. Further, since protein crosslinking was observed to occur frequently and yet amino acids only occupied ~2% of the VWF volume, it may be possible that these interactions stabilized by BS3 are specific in nature. This hypothesis is the subject of current investigations in our laboratory.

In our studies using light and neutron scattering, we did not observe changes in the $R_g$ of VWF upon addition of 1.25 M Gd-HCl or deuterated Gd-DCl. In contrast it has been noted that several smaller and more compact proteins including...
α-tryptophan synthase (38), cytochrome c (39), decorin (40), creatine kinase (41), RNaseA (42), and lysozyme (43) to name a few, change their $R_g$ upon addition of 0.3–8 mM Gd-HCl. It is perhaps not surprising that addition of Gd-HCl did not change VWF radius, because this protein is loosely packed (Table 3). A recent study suggests that denatured proteins behave as a random coil and that there exists a power law relationship between the number of amino acid residues and $R_g$ (44). Upon extrapolating the findings of this article to the 4100 amino acid ΔPro-VWF, it can be predicted that even complete denaturation of this protein is not likely to yield a greater $R_g$ value. Structural changes at length scales <10 nm were however apparent in our study. These changes at small length scales appear to increase the susceptibility of VWF to proteolytic cleavage in the presence of ADAMTS-13 similar to a previous report (8).

Previous studies have shown that fluid shear enhances the binding of VWF to platelets (45), and that it facilitates VWF proteolysis by ADAMTS-13 (9). The force applied to the protein even at the highest shear rates in these experiments is small, ~0.1 pN (46). In support of the proposition that shear causes large scale changes in protein conformation, surface-immobilized VWF has been shown to extend from a globular to an unfolded state upon application of fluid shear (47). Others suggest, however, that this observation may be substrate specific because VWF immobilized on collagen does not undergo similar changes in response to fluid flow (48). In support of the proposition that subtle changes are sufficient for functional alterations, x-ray studies show how point mutations in the A1-domain and GpIb receptor enhance VWF-GpIb binding affinity, without dramatically altering protein structure (19). We have also observed in studies that measure VWF solution structure changes in real time using SANS that the protein undergoes conformation changes at small length scales upon application of hydrodynamic stress.3 Besides, large scale conformation changes and subtle changes to individual domains, it has recently been shown that ADAMTS-13 proteolysis of VWF is enhanced following the binding of platelet GpIbα to VWF A1 domain (49). These authors suggest that the A1 domain of VWF inhibits cleavage of the A2 domain, and that this inhibition is relieved by binding with platelet GpIb. Thus, interactions between the domains of VWF may be important functional regulators of protein function. The role of Gd-HCl in our studies may thus be to relieve these interactions between the A2 and other domains, thereby facilitating the action of ADAMTS-13.

Whereas the exact nature of the arrangement of domains and their response to biochemical/physical stimulus is under investigation, the present data are consistent with a model where VWF multimers are formed by strong hydrophobic interactions between different domains of the protomers, which can keep this protein in a passive form. Fluid shear and surface immobilization may cause subtle changes in the protein structure that expose hitherto masked domains. Finally, the current article establishes small angle scattering tools for the study of blood proteins, which may be valuable in future studies of protein domain-domain interactions.

\[3\] I. Singh, L. Porcar, and S. Neelamegham, manuscript in preparation.

**References**

1. Sadler, J. E. (1998) *Annu. Rev. Biochem.* 67, 395–424
2. Kroll, M. H., Hellums, J. D., Mcintire, L. V., Schafer, A. L., and Moake, J. L. (1996) *Blood* 88, 1525–1541
3. Wagner, D. N., Olmsted, J. B., and Marder, V. J. (1982) *J. Cell Biol.* 95, 355–360
4. Savage, B., Saldivar, E., and Ruggeri, Z. M. (1996) *Cell* 84, 289–297
5. Doggett, T. A., Girdhar, G., Lawshe, A., Schmidtke, D. W., Laurenti, I. J., Diamond, S. L., and Diacovo, T. G. (2002) *Biophys. J.* 83, 194–205
6. Kasirer-Friede, A., Cozzi, M. R., Mazzucato, M., De Marco, L., Ruggeri, Z. M., and Shattil, S. J. (2004) *Blood* 103, 3403–3411
7. Levy, G. G., Nichols, W. C., Lian, E. C., Foroud, T., McClintick, J. N., McGee, B. M., Yang, A. Y., Siemieniak, D. R., Stark, K. R., Grupp, R., Sarode, R., Shirin, S. B., Chandrasekaran, V., Stabler, S. P., Sabio, H., Bouhassira, E. E., Upshaw, J. D., Jr., Ginsburg, D., and Tsai, H. M. (2001) *Nature* 413, 488–494
8. Tsai, H. M. (1996) *Blood* 87, 4235–4244
9. Tsai, H. M., Sussman, II, and Nagel, R. L. (1994) *Blood* 83, 2171–2179
10. Slattery, H., Loscalzo, J., Bockenstein, P., and Handin, R. I. (1985) *J. Biol. Chem.* 260, 8559–8563
11. Ohmori, K., Fretto, L. J., Harrison, R. L., Switzer, M. E., Erickson, H. P., and McKee, P. A. (1982) *J. Cell Biol.* 95, 632–640
12. Fowler, W. E., Fretto, L. J., Hamilton, K. K., Erickson, H. P., and McKee, P. A. (1986) *J. Clin. Invest.* 76, 1491–1500
13. Raghavachari, M., Tsai, H., Kortke-Marchant, K., and Marchant, R. E. (2000) *Colloids Surf. B. Biointerfaces* 19, 315–324
14. Shankaran, H., Alexandridis, P., and Neelamegham, S. (2003) *Blood* 101, 2637–2645
15. Loscalzo, J., Fisch, M., and Handin, R. I. (1985) *Biochemistry* 24, 4468–4475
16. Emsley, J., Cruz, M., Handin, R., and Liddington, R. (1998) *J. Biol. Chem.* 273, 10396–10401
17. Celik, R., Ruggeri, Z. M., and Varughese, K. I. (2000) *Nat. Struct. Biol.* 7, 881–884
18. Fukuda, K., Doggett, T. A., Bankston, L. A., Cruz, M. A., Diacovo, T. G., and Liddington, R. C. (2002) *Structure (Camb.)* 10, 943–950
19. Huizinga, E. G., Tsuji, S., Romijn, R. A., Schiphorst, M. E., de Groot, P. G., Sixma, J. J., and Gros, P. (2002) *Science* 297, 1176–1179
20. Calmettes, P., Roux, B., Durand, D., Desmadril, M., and Smith, J. C. (1993) *J. Mol. Biol.* 231, 840–848
21. Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., and Orkin, S. H. (1998) *Cell* 52, 229–236
22. Fischer, B. E., Schlokat, U., Reiter, M., Mundt, W., and Dorner, F. (1997) *Cell Mol. Life Sci.* 53, 943–950
23. Xiao, Z., Goldsmith, H. L., McIntosh, F. A., Shankaran, H., and Neelamegham, S. (2006) *Biophys. J.* 90, 2221–2224
24. Glinka, C. J., Barker, J. G., Hammouda, K., Krueger, S., Moyer, J. J., and Orkin, S. H. (1998) *J. Cell Biol.* 134, 1461–1468
25. Spalla, O. (2002) *Neutrons, X-Rays and Light Scattering Methods Applied to Soft Matter*, 3rd Ed., pp. 23–50, Elsevier, New York
26. Calmettes, P., Durand, D., Desmadril, M., and Smith, J. C. (1994) *Biophys. Chem.* 53, 105–113
27. Beaucage, G. (1996) *J. Appl. Crystallogr.* 29, 134–146
28. Saenko, E., Sarafanov, A., Greco, N., Shima, M., Loster, K., Schwinn, H., and Josic, D. (1999) *J. Chromatogr. A.* 852, 59–71
29. Bienkowska, J., Cruz, M., Atiemo, A., Handin, R., and Liddington, R. (1997) *J. Biol. Chem.* 272, 25162–25167

Acknowledgments—We thank Dr. Gian P. Visentin for reviewing and providing critical comments on this manuscript. We also thank Lionel Porcar for discussions about SANS experiments. The NIST neutron research facility used in this work is supported in part by the NSF under agreement DMR-0454672. We acknowledge support of the NIST, US Department of Commerce, in providing the neutron facilities used in this work.
30. Jacrot, B. (1976) Rep. Progr. Phys. 39, 911–953
31. Bendetowicz, A. V., Wise, R. J., and Gilbert, G. E. (1999) J. Biol. Chem. 274, 12300–12307
32. Ulrichs, H., Vanhoorelbeke, K., Girma, J. P., Lenting, P. J., Vauterin, S., and Deckmyn, H. (2005) J. Thromb. Haemost. 3, 552–561
33. Kuhn, W. (1934) Kolloid-Z 68, 2–11
34. Yang, Z., Kollman, J. M., Pandi, L., and Doolittle, R. F. (2001) Biochemistry 40, 12515–12523
35. Hall, C. E., and Slayter, H. S. (1959) J. Biophys. Biochem. Cytol. 5, 11–16
36. Wen, J., Arakawa, T., and Philo, J. S. (1996) Anal. Biochem. 240, 155–166
37. Savage, B., Sixma, J. J., and Ruggeri, Z. M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 425–430
38. Gualfetti, P. I., Iwakura, M., Lee, J. C., Kihara, H., Bilsel, O., Zitzewitz, J. A., and Matthews, C. R. (1999) Biochemistry 38, 13367–13378
39. Segel, D. J., Fink, A. L., Hodgson, K. O., and Doniach, S. (1998) Biochemistry 37, 12443–12451
40. Scott, P. G., Grossmann, J. G., Dodd, C. M., Sheehan, J. K., and Bishop, P. N. (2003) J. Biol. Chem. 278, 18353–18359
41. Zhou, J. M., Fan, Y. X., Kihara, H., Kimura, K., and Amemiya, Y. (1997) FEBS Lett. 415, 183–185
42. Sosnick, T. R., and Trewhella, J. (1992) Biochemistry 31, 8329–8335
43. Hoshino, M., Hagihara, Y., Hamada, D., Kataoka, M., and Goto, Y. (1997) FEBS Lett. 416, 72–76
44. Kohn, J. E., Millett, L. S., Jacob, J., Zagrovic, B., Dillon, T. M., Cingel, N., Dothager, R. S., Seifert, S., Thiyagarajan, P., Sosnick, T. R., Hasan, M. Z., Pande, V. S., Ruczinski, I., Doniach, S., and Plaxco, K. W. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12491–12496
45. Goto, S., Salomon, D. R., Ikeda, Y., and Ruggeri, Z. M. (1995) J. Biol. Chem. 270, 23352–23361
46. Shankaran, H., and Neelamegham, S. (2004) Biophys. J. 86, 576–588
47. Siedlecki, C. A., Lestin, B. J., Kottke-Marchant, K. K., Eppell, S. J., Wilson, D. L., and Marchant, R. E. (1996) Blood 88, 2939–2950
48. Novak, L., Deckmyn, H., Damjanovich, S., and Harasfalvi, J. (2002) Blood 99, 2070–2076
49. Nishio, K., Anderson, P. J., Zheng, X. L., and Sadler, J. E. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10578–10583