Solution Studies of the Quaternary Structure and Assembly of Human von Willebrand Factor†

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ABSTRACT: The reversible association of protomers of von Willebrand protein (vWF) was studied in order to analyze the forces and mechanism of vWF polymer assembly. At concentrations of vWF found in plasma (~16 μg/mL), disulfide bond reduction with 50 mM 2-mercaptoethanol (2-ME) markedly reduced both vWF activity, as measured by ristocetin-dependent platelet agglutination, and average polymer size ($\bar{R}_h$, the mean hydrodynamic radius) in solution, as determined by quasi-elastic light scattering (QLS) and by gel filtration chromatography. With increasing vWF concentration, activity and $\bar{R}_h$ increased despite reduction of interprotomer disulfide bonds. Changes in temperature after 2-ME treatment produced reversible changes in activity and $\bar{R}_h$. Varying the total vWF concentration at any given temperature after 2-ME treatment changed $\bar{R}_h$ in a consistent and predictable fashion, so that estimates of the dissociation constant for vWF protomer–polymer equilibrium were obtained: $K_d^{5°C}$ = 0.77 μg/mL, $K_d^{25°C}$ = 2.4 μg/mL, and $K_d^{37°C}$ = 7.7 μg/mL, where under the conditions of reduction presented here, the basic protomer of vWF is a dimer. Increasing ionic strength after 2-ME treatment with 1 M KCl did not change $\bar{R}_h$, while ~100 μM sodium dodecyl sulfate (SDS) or ~300 μM sodium deoxycholate (DOC) reduced both $\bar{R}_h$ and activity compared with those of unreduced polymer. These data show that disulfide bonds are necessary to maintain vWF polymer size and activity at plasma concentrations but that noncovalent forces of association can maintain vWF polymer size and activity at higher concentrations. These forces of association may be important for polymer assembly during intracellular synthesis of vWF.

The von Willebrand protein (vWF)[1] is a polymeric plasma glycoprotein (Legaz et al., 1973; Shapiro et al., 1973; Olson et al., 1977) that facilitates platelet adhesion to vascular subendothelium and is essential for normal platelet plug formation (Jorgensen & Borchgrevink, 1964; Hovig & Stormoken, 1974). This function is readily assayed in vitro by monitoring platelet agglutination in the presence of the cationic antibiotic ristocetin (Howard et al., 1973; Weiss et al., 1973). vWF is composed of disulfide-linked M_r 230 000 monomers and exists in plasma as a series of multimers of varying molecular weights (van Mourik et al., 1974; Martin et al., 1981; Ruggeri & Zimmerman, 1981). Polymer size correlates with activity in ristocetin-dependent platelet agglutination assays (Martin et al., 1981; Furlan et al., 1979; Chan & Chan, 1982).

A loss of vWF activity and a decrease in polymer size follow exposure of vWF to mercaptans (Austen, 1970; Austen et al., 1975; Kirby & Mills, 1975). Disulfide reduction decreases both vWF activity and multimer size and distribution as assessed by SDS–polyacrylamide and/or agarose gel electrophoresis (Kirby & Mills, 1975; Cooper et al., 1975; Fukui et al., 1977; Blomback et al., 1978; Counts et al., 1978; Suzuki et al., 1980). Under conditions producing complete disulfide reduction, a single protein of M_r 230 000 (≡vWF monomer) appears electrophoretically, while lesser concentrations of 2-ME produce multimers of smaller size than the native distribution (Counts et al., 1978). Because noncovalent forces of association are likely to be important in polymer assembly in vivo, we have investigated the reversible association of vWF protomers (≡dimer) under a variety of conditions using QLS and gel filtration techniques. Our data demonstrate that (1) under appropriate conditions, vWF multimers persist in aqueous solution after complete disulfide reduction, (2) these noncovalently associated multimers retain biologic activity, and (3) reversible hydrophobic interactions are an important binding force among vWF protomers following disulfide reduction and alkylation.

Materials and Methods

Materials. 2-ME, TX, iodoacetamide, DTNB, and DOC were obtained from Sigma Chemical Co., St. Louis, MO. SDS was purchased from Bio-Rad Laboratories, Richmond, CA. Iodo[^{14}C]acetamide and Na^{125}I were obtained from New England Nuclear, Boston, MA. Liquiscint was purchased from Burdick & Jackson Laboratories, Muskegon, MI. Ristocetin was obtained from Bio-Data Corp., Horsham, PA. Iodo-beads were purchased from Pierce Chemical Co., Rockford, IL. Polyvinyl chloride/propylene copolymer filters of 0.45-μm pore size were obtained from Millipore Filter Corp., Bedford, MA. Sephacryl S-1000, Sepharose 4B, and Sephadex G-25 were

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[1] Abbreviations: vWF, von Willebrand protein; 2-ME, 2-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DOC, deoxycholate; SDS, sodium dodecyl sulfate; TBS, 10 mM Tris, pH 7.8, and 0.15 M NaCl; QLS, quasi-elastic light scattering; Tris, tris(hydroxymethyl)-aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; TX, Triton X-100.

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purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other materials used were of reagent grade or better.

γ emission was assayed by using a Beckman Model 8000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). Liquid scintillation counting was performed on a Tracor Analytic Model 43 liquid scintillation counter (Tracor Analytic, Inc., Elk Grove Village, IL).

vWF Preparation. vWF was prepared from a cryoprecipitate of plasma as described in an earlier publication (Loscalzo & Handin, 1984).

Radioiodination of vWF. One Iodo-bead was incubated with 0.5–1.0 mCi of $Na^{125}I$ (in 0.1 N NaOH) at 25 °C for 15 min. To this was added 1.0 mL of 0.1 mg/mL vWF in 10 mM Tris, pH 7.8, and 0.15 M NaCl (TBS), and the incubation was continued for 25 min with gentle rocking. The protein solution was then applied to a 10 × 0.7 cm Sephadex G-25 column which had been developed with 1 mL of 5 mg/mL bovine serum albumin in TBS. The column was then developed with TBS; 12 0.3-mL fractions were collected and assayed for total and 25% trichloroacetic acid precipitable radioactivity. Routinely, column fractions 5–7 contained maximal protein-bound counts with a specific activity of ~0.1 μCi/μg. These fractions were either used immediately or stored in 50% glycerol at −30 °C for up to 4 weeks without any appreciable loss of protein-bound radioactivity.

Formalin-Fixed Platelets. Formalin-fixed platelets (Brinkhaus & Read, 1978) used in ristocetin agglutination assays (see below) were prepared by mixing an equal volume of 10 mM Tris, pH 7.8, 0.15 M NaCl, and 2% paraformaldehyde with freshly prepared platelet-rich plasma in 0.014 M sodium citrate and incubating for 1 h at 37 °C. Platelets were then either immediately washed or stored at 4 °C for up to 16 h after which they were washed twice with 10 mM Tris, pH 7.8, 0.15 M NaCl, and 20 mM EDTA and then twice with TBS. Platelets were resuspended at $4 \times 10^5/\mu$L and stored at 4 °C for up to 3 weeks or at −30 °C for up to 2 months (Allain et al., 1975).

vWF Activity. The ristocetin-dependent agglutination of formalin-fixed platelets by vWF was used as a measure of activity (Howard & Firkin, 1971; Olson et al., 1975) as described in detail in an earlier publication (Loscalzo & Handin, 1984).

Sulfhydryl Analysis. Disulfide groups of the native protein were reduced by addition of 50 mM 2-ME to 0.1 mg/mL vWF in TBS for 1 h at 25 °C. Carboxamidation of the reduced sulfhydryl groups was accomplished with 60 mM iodoacetamide in the same buffer and the pH maintained by careful titration with dilute NaOH. These reduction conditions repeatedly exposed 125 of the 151 total (Legaz et al., 1973) sulfhydryl groups per monomer (all of which are disulfide linked in the native protein).

In kinetic experiments, the rate of appearance of sulfhydryl groups after disulfide reduction was analyzed with DTNB (Ellman, 1959; Gabeeb, 1966). After addition of 2-ME to a final concentration of 50 mM, 0.4-mL aliquots of a 4-mL incubation solution of 0.1 mg/mL vWF were removed at various times, and the protein was precipitated with 25% ice-cold trichloroacetic acid (v/v). Each sample was centrifuged at 25 °C in a Beckman microfuge (Backman Instruments, Inc., Fullerton, CA) at 8700*g* for 4 min. The pellet was washed 3 times with 25% ice-cold trichloroacetic acid and redissolved in 1 mL of 10 mM Tris, pH 7.8, 0.15 M NaCl, 8 M urea, and 5% SDS. To 0.8 mL of this solution was added 0.2 mL of 0.5 mg/mL DTNB. After 20 min, the absorbance at 410 nm was measured and adjusted by subtracting the absorbance of reagent and protein blanks. A molar extinction coefficient of 14 200 M^{-1} cm^{-1} for the nitrothiophenolate ion was used to calculate the concentration of free sulfhydryl groups (Collier, 1973). At various times during the incubation, the vWF activity of the incubation solution was assayed by using 0.01 mg/mL final concentration of vWF.

Quasi-Elastic Light Scattering Measurements. QLS measurements were performed at a scattering angle of 90° to obtain the mean diffusion coefficient ($\bar{D}$) of vWF in solution over a range of temperatures. Measurements were also performed at other angles to ensure that polymer–polymer interactions were not important. A Spectra Physics argon ion laser (λ 488 nm) was used in conjunction with an EMI photomultiplier tube (Model 9863) and with either a 56-channel autocorrelator (Model 6864, Nicomp Instruments, Santa Barbara, CA) or a 72-channel autocorrelator (Model 1096, Langley-Ford Instruments, Amherst, MA). Data were analyzed by the method of cumulants (Koppel, 1972) on an MF-211 microcomputer (Charles River Data Systems, Natick, MA). From $\bar{D}$, the mean hydrodynamic (Stokes') radius, $\bar{R}_h$, was determined (Einstein, 1956) from the equation:

$$\bar{R}_h = kT/(6\pi\eta\bar{D}) \quad (1)$$

where k is Boltzmann's constant, T is the absolute temperature, and η is the solvent viscosity. All reagent solutions were filtered through 0.45-μm Millipore filters, and all protein solutions were centrifuted at 8000*g* for at least 15 min at 25 °C to remove dust and aggregated material. The sensitivity of this system is such that 25-Å monodispersed particles at 1 mg/mL are readily measurable with a polydispersity of <10%.

Chromatographic Analysis. All chromatographic analyses of diluted, reduced, and/or detergent-treated samples of vWF were performed on 8–10 × 0.7 cm columns of Sephacryl S-1000 at 25 °C. In most cases, 0.5-mL samples were applied to each column and 0.2-mL fractions collected.

The molecular weight of the protomer of vWF prepared by disulfide reduction and carboxamidation as described above was estimated both by gel electrophoresis of denatured species (see below) and by Sepharose 4B column chromatography in TBS at 25 °C using concentrations of (radioiodinated) vWF sufficiently dilute to prevent noncovalent association (i.e., 5 μg/mL, with a specific activity of 10 061 cpm/μg). Horse spleen ferritin, bovine thyroglobulin, purified fibronectin, and catalase were used as standards.

Gel Electrophoresis. Samples of vWF were analyzed in the presence and absence of 2-ME on SDS gels containing 4% polyacrylamide or 2% agarose. Gel electrophoresis was performed by the methods of Weber & Osborne (1969), as modified by Laemmli (1970), and of Ruggeri & Zimmerman (1981) using purified fibronectin (both native and reduced) and dimethyl suberimidate cross-linked IgM as standards.

Protein Determinations. vWF protein concentrations were estimated according to the method of Lowry and colleagues (1951).

Results

vWF Specific Activity. The elution profile of vWF on a Sephacryl S-1000 column is shown in Figure 1. The bulk of protein elutes after the column void volume (beyond ~290 mL) and is primarily comprised of fibrinogen. A small fraction of the applied protein elutes early, at the void volume, contains vWF activity, and migrates as a single, M_r 230 000 band on denaturing SDS–polyacrylamide gels after extensive 2-ME treatment. While the peak containing vWF activity is relatively symmetric, the specific activity is skewed to the left. The observation that early eluting fractions have higher specific

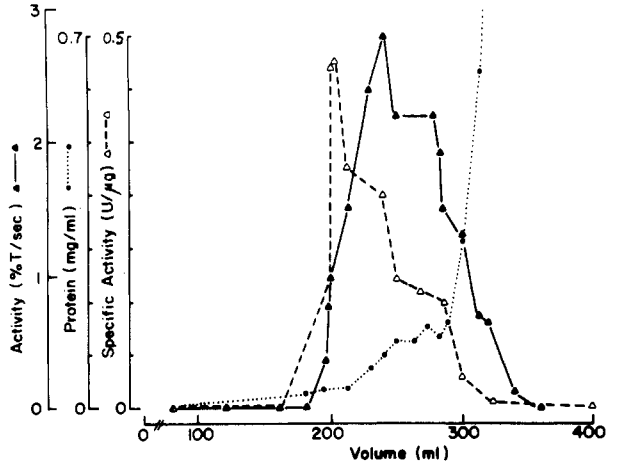

FIGURE 1: Elution profile of vWF on Sephacryl S-1000. To a 99 × 2.5 cm column was applied 10 mL of a polyethylene glycol fraction of a plasma cryoprecipitate prepared as described under Materials and Methods. The column was developed at 25 °C with 50 mM Tris, pH 7.4, 0.15 M NaCl, and 0.02% NaN_3 at 70 mL/h. Four-milliliter fractions were collected. Protein concentrations (closed circles), vWF activity (closed triangles), and specific activity (open triangles) were determined for each fraction as described under Materials and Methods.

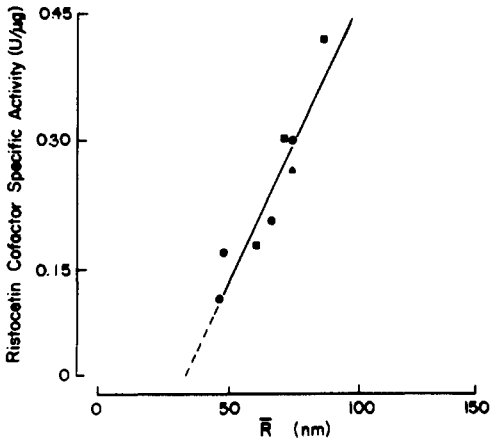

FIGURE 2: Specific activity of column fractions as a function of Stokes' radius, $\bar{R}_h$. Specific activity and $\bar{R}_h$ were determined as described under Materials and Methods at 25 °C in column buffer. The three symbols represent three different preparations of vWF. Each sample contained 0.08–0.12 mg/mL protein.

activities in ristocetin-dependent platelet agglutination assays is consistent with previous observations that larger vWF polymers have enhanced biologic activity (Furlan et al., 1979; Chan & Chan, 1982). Unless otherwise indicated, all experiments were performed with those column fractions having the greatest specific activity (i.e., fractions eluting between 200 and 212 mL).

QLS Analysis of Column Fractions. Estimates of the mean diffusion coefficients and the derived Stokes' radii of several column fractions were determined by QLS analysis. Figure 2 shows the correlation of specific activity with mean Stokes' radius for several column fractions and suggests that vWF activity may be related not only to electrophoretic mobility under denaturing conditions but also to the mean hydrodynamic radius of the multimer population in solution. The abscissal intercept of this plot also suggests that the minimal hydrodynamic radius that will support ristocetin-dependent platelet agglutination is ~330 Å.

Disulfide Bonds and vWF Activity. The effect of disulfide reduction with 50 mM 2-ME on vWF activity and on titratable sulfhydryl groups of vWF protein is shown in Figure 3. vWF

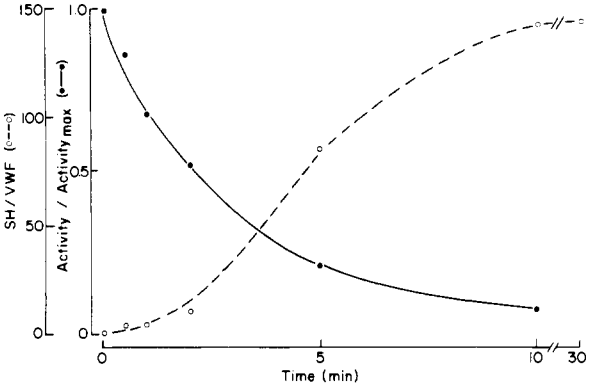

FIGURE 3: Time course of sulfhydryl group reduction and vWF activity after treatment with 2-ME. Sulfhydryl group analysis (open circles) and vWF activity (closed circles) were determined as described under Materials and Methods.

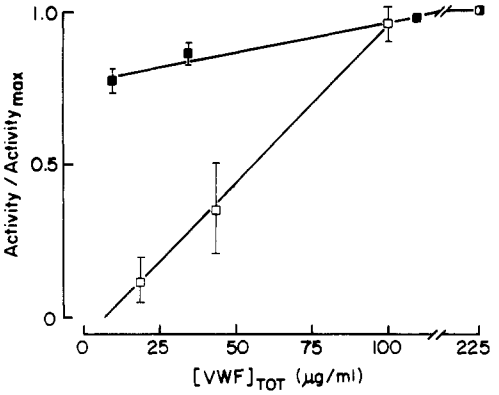

FIGURE 4: Effect of sulfhydryl reduction on vWF activity at varying vWF concentrations. vWF at 225 μg/mL was incubated with (open squares) or without (closed squares) 50 mM 2-ME for 1 h at 25 °C. Iodoacetamide (60 mM) was then added, the pH was adjusted, the vWF solutions were diluted to the concentrations indicated on the abscissa, and vWF activity was determined at each of these concentrations as described under Materials and Methods. Each point represents the average of two or three experiments performed in triplicate.

at 100 μg/mL was exposed to 2-ME, and aliquots were removed at the times indicated in the figure for the determination of titratable sulfhydryl groups and vWF activity; in all cases, vWF activity was measured at a final concentration of 15 μg/mL. Incubating vWF with 2-ME at two different concentrations (15 and 50 μg/mL) produced identical results. These data suggest that the reduction of a small number of disulfide bonds decreases vWF activity by 50% when concentrations of vWF found in plasma (~16 μg/mL) are used and that, under these conditions of reduction, ~125 of the 151 available sulfhydryl groups are reduced. In order to investigate the noncovalent forces that maintain (polymeric) quaternary structure of vWF, the persistence of vWF activity after disulfide reduction was examined as a function of vWF concentration. The data depicted in Figures 3 and 4 show that vWF activity is maintained following reduction and carboxamidation of ~125 of the available 151 sulfhydryl groups at vWF concentrations approximately 5-fold greater than that of plasma. The activity of 2-ME-treated vWF decreases relative to that of native vWF with dilution, suggesting that concentration-dependent, noncovalent interactions among vWF protomers can maintain polymeric structure after 2-ME treatment.

Disulfide Bonds, Multimer Size, and Protomer Identity. In order to demonstrate by another method that reversible forces of association maintain polymeric structure after di-

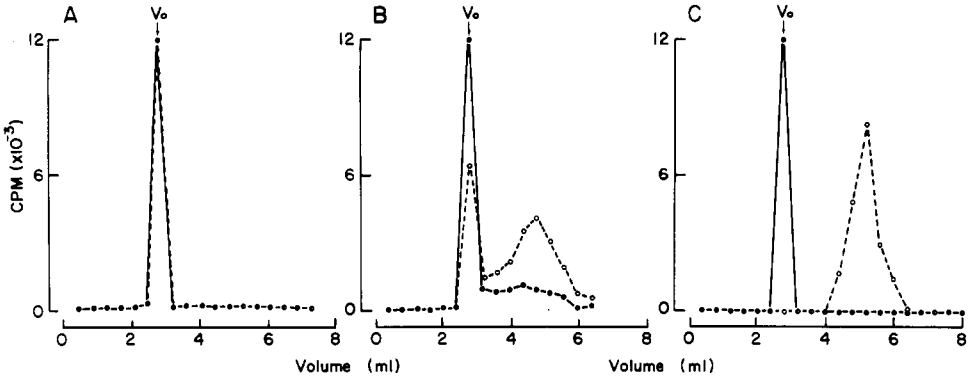

FIGURE 5: Sephacryl S-1000 elution profiles of vWF: effect of 2-ME and dilution. (A) To an 8 × 0.75 cm column of Sephacryl S-1000 in TBS was applied 0.4 mL of 120 μg/mL vWF with (open circles) and without (closed circles) 50 mM 2-ME/60 mM iodoacetamide. Fractions of 0.4 mL were collected and analyzed for trichloroacetic acid precipitable radioactivity. Each initial sample contained 1050 cpm/μg of protein. (B) Identical experiment applying 0.4 mL of 12 μg/mL vWF exposed at this same concentration to 50 mM 2-ME/60 mM iodoacetamide. Each initial sample contained 10661 cpm/μg of protein. (C) Identical experiment as (A) with the chromatographic medium containing 0.1% SDS.

sulfide reduction with 2-ME under the conditions of these experiments, gel chromatography with Sephacryl S-1000 was used. Figure 5A shows the elution pattern for 120 μg/mL vWF before and after exposure to 2-ME. At this concentration, 2-ME treatment failed to alter the elution position of vWF, with the bulk of protein eluting at or near the void volume. With a 10-fold dilution, however, the elution pattern changed (Figure 5B), and a later eluting component appeared well within the column volume. Exposure of 2-ME-treated vWF to 3.5 mM SDS (0.1%) shifted 95% of the protein to the later eluting peak (Figure 5C), suggesting that treatment with 50 mM 2-ME reduced many of the available (interchain) disulfide bonds. To characterize the extent of reduction of disulfides and to define the protomer of vWF involved in these reversible associations, SDS–4% polyacrylamide, as well as 2% agarose, gel electrophoresis was performed on the 2-ME-treated, carboxamidated vWF used in these experiments. Ninety-five percent of the total protein was found to have an electrophoretic mobility corresponding to a molecular weight of approximately 450000, i.e., a dimer of the basic vWF monomer. In addition, Sepharose 4B column chromatography under nondenaturing conditions (TBS) yielded a very similar value, i.e., 480000 daltons (see Materials and Methods).

Temperature and Multimer Size. In order to study further these noncovalent forces of association, the temperature of the system was varied and the average diffusion coefficient of the multimer population measured by QLS methods. The average Stokes' radius derived from the diffusion coefficient of a 0.1 mg/mL solution of either native or 2-ME-treated vWF as a function of temperature is shown in Figure 6. These data show that the change in hydrodynamic size of 2-ME-treated vWF in solution is reversible within a temperature range of 5–37 °C. In contrast, heating the protein to 55 °C followed by cooling to 5 °C failed to increase $\bar{R}_h$, suggesting that protein denaturation occurred with this more extreme temperature exposure. Concomitant with this hydrodynamic size change, vWF activity also changed in a reversible fashion over the same temperature range (5–37°C) (Figure 7). Again, heating to 55 °C irreversibly denatured both native and 2-ME-treated vWF.

The average diffusion coefficient of 2-ME-treated vWF was also measured as a function of vWF concentration at three different temperatures (Figure 8). These data show that, with increasing temperature and with decreasing concentration at any given temperature, the average diffusion coefficient of a solution of 2-ME-treated vWF increases, which implies a decrease in average multimer size.

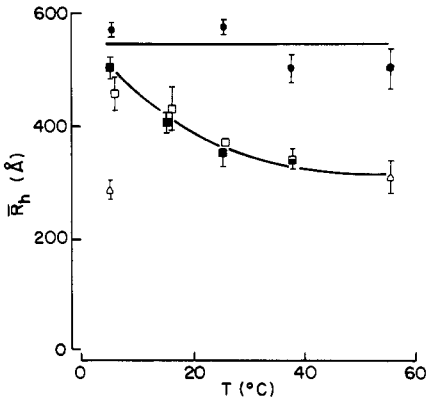

FIGURE 6: Effect of temperature on average Stokes' radius of vWF. The mean Stokes' radius (R_h) of 1.0-mL solutions of 0.1 mg/mL native (circles) and 2-ME-treated (squares) vWF in TBS was determined with increasing (closed symbols) and decreasing (open symbols) temperature from 5 to 37 °C. At the end of the experiment, 2-ME-treated samples were heated to 55 °C and then cooled to 5 °C (open triangles). All temperatures were accurate to 0.1 °C. Each point represents the average of three to seven experiments, with a polydispersity measured from the variance of 20–42% of the mean. The SEM is indicated for each point.

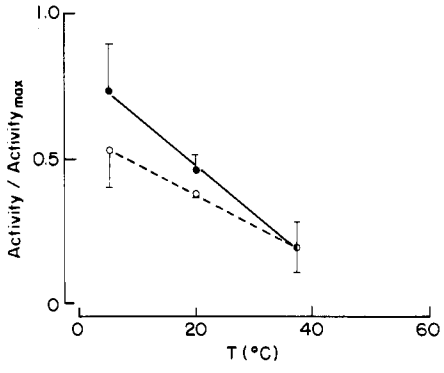

FIGURE 7: Effect of temperature on vWF activity. Native and 2-ME-treated vWF at 0.1 mg/mL in TBS were incubated at increasing (closed circles) and decreasing (open circles) temperatures for 15 min, after which 0.1 mL of each of which was added to 0.3 mL of 3 × 10^5 formalin-fixed platelets/μL. At the same temperature as that of incubation, vWF activity was measured by adding 1 mg/mL ristocetin (final concentration) and monitoring the maximal rate of change of light transmittance of the solution. The ordinate is expressed as the ratio of 2-ME-treated vWF activity to native vWF activity at each temperature.

Calculation and Analysis of Equilibrium Constants for Polymerization. The data presented in Figure 8 can be used

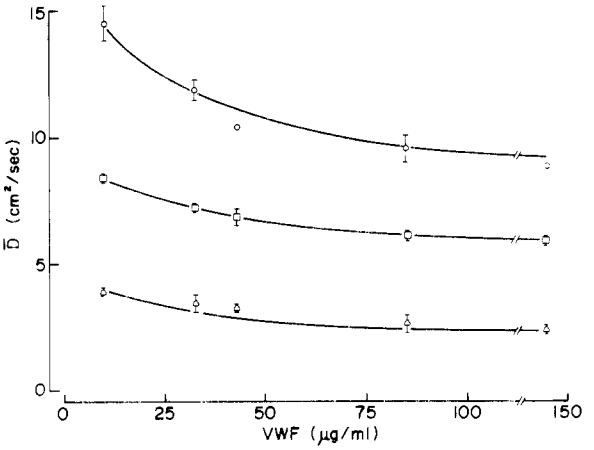

FIGURE 8: Effect of temperature and concentrations on average diffusion coefficient of vWF. Over a range of concentrations, the average diffusion coefficient of 1.0-mL samples of 2-ME-treated vWF was measured at 5 (triangles), 25 (squares), and 37 °C (circles) as described under Materials and Methods. All temperatures were accurate to 0.1 °C. At each temperature, the average diffusion coefficient of native vWF did not change with changes in concentration (data not shown). Each point represents the average of three to five determinations, with a polydispersity measured from the variance of 20–45% of the mean. The solid lines represent the theoretical fit of the data by using $K_d^{5°C} = 0.77$ μg/mL, $K_d^{25°C} = 2.4$ μg/mL, and $K_d^{37°C} = 7.7$ μg/mL as outlined under Results.

to determine the equilibrium constants for vWF protomer–polymer association. This analysis assumes that (1) vWF protomers polymerize linearly and (2) the association of protomers with linear polymers may be expressed as a simple equilibrium and need not be expressed as a cooperative, "condensation-type" polymerization process. The first assumption is consistent with the electron microscopic studies of Ohmori and colleagues (1982), while the second is based on simplicity and consistency with the data.

The equilibrium between linear chain polymers (P_M) and protomers (S) may be expressed as

$$P_M + S \rightleftharpoons P_{M+1} \tag{2}$$

Applying the law of mass action for a simple equilibrium to this reaction gives

$$[P_{M+1}] = K[P_M][S] \tag{3}$$

where K is the association constant for protomer–polymer binding. By induction, this may be generalized for polymers of any length and expressed in terms of the concentration of free protomers:

$$[P_N] = (K[S])^{N-1}[S] \tag{4}$$

Equation 4 states that the concentration of polymers is a geometrically decreasing function of polymer length.

The total concentration of protomers, both free and incorporated into polymers, is given as

$$[S_{TOT}] = \sum_N N[P_N] \tag{5}$$

By expanding the summation of eq 5 and substituting eq 4, we obtain

$$[S_{TOT}] = [S]/(1 - K[S])^2 \tag{6}$$

Rearranging eq 6 gives an expression for the concentration of free protomer, [S], as a function of total protomer concentration:

$$[S] = \{1 + [1 - \sqrt{1 + 4K[S_{TOT}]}]/(2K[S_{TOT}])\}/K \tag{7}$$

Analysis of the experimentally measured autocorrelation function of the scattered light at an angle of 90° using the method of cumulants (Koppel, 1972) allows one to measure both the mean diffusion coefficient, $\bar{D}$, and the width of the distribution of diffusion coefficients, σ, where

$$\bar{D} \equiv \int_0^\infty Dw(D)\, dD = \sum_N w_N D_N \tag{8}$$

$$\sigma \equiv \left[\int_0^\infty w(D)(D - \bar{D})^2\, dD\right]^{1/2} = [\sum_N w_N(D_N - \bar{D})^2]^{1/2} \tag{9}$$

and w_N is the normalized distribution function of the diffusion coefficients as a function of the number of protomers, N.

To interpret the results of the QLS data, we calculated the theoretical distribution, $w(D)$, for each value of S_{TOT} assuming a given K. Assuming a normal distribution, w_N is given by

$$W_N = N^2P_NF_N/\sum_N N^2P_NF_N \tag{10}$$

where P_N is the concentration of a polymer composed of N protomers and F_N is the particle-scattering factor given explicitly for thin cylinders by (Jullien & Thusius, 1976)

$$F_N = |\tfrac{1}{2}\int_{-1}^{1}\{[\sin(QL_Nx/2)]/(QL_Nx/2)\}\, dx|^2 \tag{11}$$

In this equation, L_N is the length of a polymer composed of N protomers and Q is the magnitude of the scattering vector, expressed as

$$Q = (4\pi\mathcal{N}/\lambda)\sin(\theta/2) \tag{12}$$

where $\mathcal{N}$ is the refractive index of the solution, λ is the wavelength of incident light, and θ is the scattering angle.

The diffusion coefficient for a polymer of any length is given by

$$D = kT/f \tag{13}$$

where k is Boltzmann's constant, T is the absolute temperature, and f is the average translational friction factor of the particle. For a prolate ellipsoid of revolution of semimajor axis, a, and semiminor axis, b, f is given by Perrin (1936) as

$$f = 6\pi\eta R_0\{(z^2 - 1)^{1/2}/z^{1/3}\ln[z + (z^2 - 1)]^{1/2}\} \tag{14}$$

where $z = a/b$, the axial ratio, $R_0 \equiv (ab^2)^{1/3}$, and η is the shear viscosity of the solvent.

The actual analysis is performed by a reiterative procedure. First, the value of $[S_{TOT}]$ is fixed on the basis of the solution concentration of vWF and an assumed protomer size. The concentration of each polymeric species is then calculated from eq 4. The weighting factors for each polymer are then calculated by using this result and eq 10–12 with $\theta = 90°$. The diffusion coefficient for each polymer composed of N protomers is then calculated by solving eq 13 and 14. Finally, the weighting factors and diffusion coefficients for each N-mer are combined and the mean diffusion coefficient and variance derived from eq 8 and 9.

Assuming that vWF polymers are made up of linear molecules of protomer size, 500 Å × 25 Å (Ohmori et al., 1982), and of average molecular weight, 450 000, the above theoretical analysis may be performed for any value of K, the dissociation constant for the vWF protomer–polymer equilibrium. When the values $K_d^{5°C} = 0.77$ μg/mL, $K_d^{25°C} = 2.4$ μg/mL, and $K_d^{37°C} = 7.7$ μg/mL are used, an excellent fit to the experimental data is obtained, as shown by the solid curves of Figure 8, with a polydispersity determined from the variance of 20–45% of the mean. This value of polydispersity is nearly identical with that experimentally measured. Table I lists the theoretical mean degree of polymerization calculated at four different total concentrations of vWF at three different temperatures. From these data, one may estimate the mean

Table I: Mean Degree of Polymerization of vWF[a]

	[vWF] (μg/mL)			
T (°C)	9.4	18.6	46.0	87.0
5	7-mer	10-mer	16-mer	22-mer
25	4-mer	6-mer	9-mer	12-mer
37	2-mer	4-mer	5-mer	7-mer

[a] These values represent the main number of 230 000-dalton polypeptides associated in the polymeric state at the given temperatures and concentrations of total vWF. Values were calculated by using eq 4 and 10–14 and fit the experimental data of Figure 8 best by using $K_d^{5°C}$ = 0.77 μg/mL, $K_d^{25°C}$ = 2.4 μg/mL and $K_d^{37°C}$ = 7.7 μg/mL assuming the protomer involved in polymerization is a dimer (2-mer).

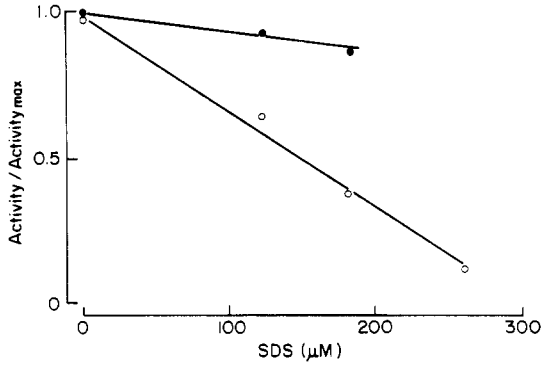

FIGURE 9: Effect of SDS on native and 2-ME-treated vWF activity. A total of 0.2 mL of native (closed circles) or 2-ME-treated (open circles) vWF was added to 0.3 mL of 2 × 10^5 formalin-fixed platelets/μL and incubated for 5 min at 37 °C with increasing concentrations of SDS in TBS. vWF activity was measured by adding ristocetin to 1 mg/mL final concentration and monitoring the maximal rate of change of light transmittance of the solution.

diffusion coefficient of the basic protomer by extrapolating the curves of Figure 8 to the y intercept. At 25 °C, this value is 1.7 × 10^{-7} cm^2/s.

The energetics of vWF protomer polymerization may be derived from these data, as well. At 25 °C, $\Delta G°$ = −10.9 kcal/mol. By use of the van't Hoff expression, $\Delta H°$ is found to be −9.0 kcal/mol. ΔS is then calculated to be 6.4 cal mol^{-1} K^{-1}. These values suggest that polymerization of vWF protomers is energetically favored on both enthalpic and entropic grounds.

Noncovalent Forces among vWF Protomers. In order to assess the nature of the noncovalent forces of association among protomers in the vWF polymer, the ionic strength and hydrophobicity of the aqueous medium were varied, and the chromatographic elution pattern, vWF activity, and mean diffusion coefficient (measured by QLS) in solutions of native and 2-ME-treated vWF were analyzed. Varying the ionic strength from 0.005 to 1.0 did not alter the elution pattern, the mean scattered light intensity, or the mean diffusion coefficient of 100 μg/mL solutions of 2-ME-treated vWF compared with those of native vWF.

Hydrophobic forces were perturbed with low concentrations of the detergent amphiphiles, SDS, deoxycholate (DOC), and Triton X-100 (TX). Concentrations of SDS as low as 50 μM decreased the biologic activity of 2-ME-treated vWF compared to similar concentrations of unreduced vWF (Figure 9). The effect of SDS and DOC on the mean Stokes' radius ($\bar{R}_h$) of native and 2-ME-treated vWF was then studied. The amphiphiles increased the diffusion coefficient and, thus, decreased the calculated Stokes' radius of 2-ME-treated vWF, as shown in Figure 10.

The reversibility of vWF inactivation after exposure to DOC was also examined. 2-ME-treated vWF and native vWF were exposed to 100 μM DOC, and the difference in vWF activity

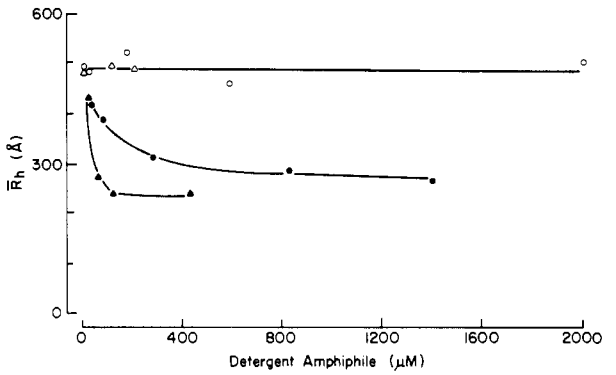

FIGURE 10: Effect of detergent amphiphiles on average Stokes' radius of vWF. To 1.0 mL of 0.1 mg/mL native (open symbols) or 2-ME-treated (closed symbols) vWF was added increasing concentrations of SDS (triangles) or DOC (circles) at 25 °c, and the mean Stokes' radius ($\bar{R}_h$) was measured as described under Materials and Methods.

Table II: Reversibility of Deoxycholate-Induced Loss of vWF Activity[a]

	activity/$activity_{max}$
no deoxycholate	1.0 ± 0.05
200 μM deoxycholate	0.36 ± 0.08
after gel filtration	0.83 ± 0.10

[a] To 1.0 mL of 0.1 mg/mL vWF in TBS with or without 50 mM 2-ME/60 mM iodoacetamide was added 0.04 mL of 5 mM deoxycholate in 0.1 M borate buffer, pH 9.0. The vWF activity of the samples before and after deoxycholate addition was determined as described under Materials and Methods. A total of 0.5 mL of each sample was then applied to a 0.5 × 1 cm column of Sephadex G-25 in TBS and the first 0.5 mL volume collected. vWF activity of these gel-filtered samples was measured. The activities listed are expressed as the ratio of activity of 2-ME-treated to native vWF samples under each set of conditions and represent the average of three experiments done in duplicate.

was recorded. Each sample was then separated from excess detergent by Sephadex G-25 chromatography, with or without 50 mM 2-ME at 24 °C, and vWF activity reassessed (Table II). Gel filtration of DOC-treated vWF on Sephadex G-25 produced recovery of most of the vWF activity. These data suggest that vWF subunits are held together in part by hydrophobic forces that are readily perturbed and disrupted by low concentrations of detergent amphiphiles in a nondenaturing and fully reversible fashion.

Discussion

Most investigators currently believe that vWF polymers are formed by covalent disulfide linkage of lower molecular weight protomers (Austen, 1970; Austen et al., 1975; Kirby & Mills, 1975; Cooper et al., 1975; Fukui et al., 1977; Blomback et al., 1978; Counts et al., 1978; Suzuki et al., 1980). Several reports have implied, however, that noncovalent forces may also play a role in vWF protomer association. Van Mourik and colleagues (1974) observed dissociation of high molecular weight polymers without disulfide reduction after dialysis against solutions of low pH and ionic strength. Counts and associates (1978) observed dissociation of high molecular weight polymers on storage in 1% SDS for several days in the absence of disulfide reducing agent. In addition, these investigators showed that there was no difference in the distribution of dimethyl suberimidate cross-linked multimers between native vWF and 2-ME-treated vWF cross-linked at the same concentration. Chan & Chan (1982) have shown that sonication of vWF polymers produces smaller sized multimers, and we have shown that the effect of sonication is reversible in a time-dependent manner (unpublished observation). The data

presented here demonstrate the importance of noncovalent forces of association in maintaining the polymeric state of vWF. While our data may be interpreted to suggest that interprotomeric disulfide bonds maintain polymeric structure, in the context of these other studies, another interpretation is simply that intraprotomeric or, even, intramonomeric disulfide bonds maintain polymeric structure by imparting the appropriate conformational restraints on the polypeptide to support strong, noncovalent hydrophobic associations.

In the present study, the vWF activity that persists after 2-ME-treatment and decreases after dilution of the protein could be explained by a fraction of unreduced polymer which remains after reduction and carboxamidation. However, addition of SDS after 2-ME treatment shifts 95% of the vWF from the void volume to well within the Sephacryl S-1000 column volume, suggesting that under the worst circumstances only 5% of the total protein may be present as disulfide-linked higher order polymer. Five percent contaminating polymer in a 225 μg/mL solution of reduced carboxamidated vWF is not sufficient to account for the observed vWF activity. Hence, the decreasing vWF activity with increasing dilution observed after 2-ME treatment must be due to the disruption of noncovalent interactions among vWF polymers.

Temperature has a greater effect than concentration on $\bar{D}$ for any given change in vWF activity. This argues that the enthalpy contribution to polymerization is significant. With concentrations of vWF in excess of that found in plasma, the change in $\bar{D}$ was found to be small at any given temperature. This may be a reflection of the dependence of the mean diffusion coefficient on the product of the number of particles of a given size times the square of the number of subunits in each particle (see eq 10). Hence, even low concentrations of higher order N-mers contribute significantly to the mean diffusion coefficient of solutions of vWF. The discrepancy between loss of vWF activity and increase in $\bar{D}$ after 2-ME treatment may relate to the fact that biologic activity is approximately linearly related to polymer length (from Figure 2 and eq 14), while $\bar{D}$ is a weighted sum of the inverse of the square of particle size.

Detergent amphiphiles reversibly dissociate vWF protomers, indicating the importance of hydrophobic forces in maintaining polymeric structure. It is not yet clear whether the mechanism for this effect at such low concentrations of detergent is simply direct disruption of hydrophobic bonds and/or perturbation of secondary and/or tertiary structure of the polypeptide at a site distant from the hydrophobic interface(s), thereby inducing long-range changes in protein conformation and protomer dissociation. Studies are currently under way to address this interesting issue.

Although the addition of detergent amphiphiles that perturb hydrophobic interactions induces depolymerization, the thermodynamic calculations derived from the temperature dependence of the equilibrium constants do not support hydrophobic interactions as the sole force of association among vWF protomers. If hydrophobic bonds alone maintained polymeric structure after disulfide reduction, little, if any, temperature dependence of K would have been noted and a much stronger ΔS term would have been derived. In addition, the fact that we find a negative enthalpic contribution to protomer association suggests that other, nonhydrophobic forces are also important in maintaining quaternary structure. Despite the fact that at high ionic strength no significant change in Stokes' radius was noted by QLS measurements, ionic bonds may nonetheless be important in maintaining quaternary structure. Because of the ionic strength dependence of the vWF activity assay itself, the function of these nondissociated polymers could not be assessed. It is possible that high ionic strength did, in fact, disrupt noncovalent ionic interactions among protomers and, thereby, unmask otherwise inaccessible hydrophobic sites on the protomer surface. Once exposed, these sites would then promote protomer association, producing polymers with a different, but nonfunctional, quaternary structure. In addition, the contribution of van der Waals forces and hydrogen bonding was not determined in these studies.

Despite these limitations, the data presented here provide firm evidence that (1) at concentrations of vWF exceeding that of plasma, polymers persist in aqueous solution after extensive disulfide reduction, (2) vWF protomers equilibrate with polymers in a temperature-dependent manner, dissociating readily with increasing temperature, (3) these polymers have biologic activity, and (4) hydrophobic forces play a role in vWF subunit assembly, the size of which subunits is consistent with that of a dimer of the basic vWF polypeptide chain.

The identity of the vWF protomer as a dimer is based on several lines of evidence. Our resulting with low porosity SDS gel electrophoresis are supported by the studies of Counts and colleagues (1978) and of Perret and colleagues (1979). Using similar methods, other investigators (Meyer et al., 1980; Ruggeri & Zimmerman, 1980, 1981; Hoyer & Shainoff, 1980) suggest that the protomer is a tetramer of 230 000-dalton polypeptide chains. The difficulty with all of these electrophoretic analyses is that the conditions of disulfide reduction were not identical in all studies nor was carboxamidation used in all cases; furthermore, proteins which are not fully reduced may behave anomalously (Ferguson, 1964) as may glycoproteins (of which vWF is one) (Bretscher, 1971), thereby making accurate molecular weight determinations difficult.

Using a low enough concentration of vWF to prevent noncovalent association among protomers (a concentration determined by the QLS data), we were able to estimate the molecular weight of the vWF protomer by column chromatography under nondenaturing conditions. By this method, we obtained a very similar value as that determined by electrophoresis under denaturing conditions. That these two different methods provide similar results under two different extremes of solvent conditions lends some additional support to the choice of dimer as the protomeric species of importance. While we cannot absolutely exclude the possibility that the protomer is a tetramer, our data do not suggest this possibility and, furthermore, using a tetramer in the equilibrium modeling analysis failed to fit the experimental data. Thus, we feel that in our hands under the conditions of sulfhydryl reduction and carboxamidation described here, the protomer of vWF is a dimer.

The calculations of polymerization constants use a model of elongated protomers assembling end-to-end in a noncooperative fashion. Such a model fits the data extremely well (Figure 8), thus giving credibility to its simple assumptions. Ohmori and colleagues (1982) have suggested that, with progressive disulfide reduction, vWF becomes more nodular on electron micrographs. Shape changes were not included in our model because the data fit the simpler case so well. Given the strong dependence of the translational friction factor, f, on axial ratio, marked shape changes would be expected to influence the calculations significantly. Since the simple model fits the data obtained from vWF in solution so well, the possibility that the shape changes seen on micrographs do not occur in solution must be raised.

At the concentrations of human vWF found in plasma, our chromatographic and QLS measurements suggest that a sig-

nificant fraction of the protein would dissociate into protomers if disulfide linkages did not exist. A theoretical analysis of actual polymer distributions based on eq 4 and the values derived from the model described by eq 8–14 confirms that at plasma concentrations of vWF (~16 μg/mL) fully 50% of 2-ME-treated vWF is present as low molecular weight species at 37 °C. It is well established that the ability of vWF to agglutinate platelets is directly dependent on its polymeric size. Thus, disulfide linkage permits adequate amounts of functional polymeric vWF to exist at total vWF concentrations which are too low to favor noncovalent polymer assembly. Intracellularly, however, the local concentration of vWF at the site of synthesis in the endothelial cell or platelet may be sufficiently high to permit noncovalent protomer assembly and thereby favor disulfide bond formation prior to secretion. In support of this possible mechanism, several investigators have noted that vWF is secreted by human umbilical vein endothelial cells as a series of biologically active multimers (Lynch et al., 1982). Thus, it is likely that within the endothelial cell sufficiently high concentrations of vWF protomer exist to permit noncovalent assembly. Disulfide linkage may thus occur as an intracellular event prior to secretion, perhaps involving a sulfhydryl–disulfide exchange mechanism utilizing the glutathione system (Scheele & Jacoby, 1982; Gilbert, 1982).

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Registry No. vWF, 9001-27-8; DOC, 83-44-3; SDS, 151-21-3.

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