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DEFINITION OF THE AFFINITY OF BINDING BETWEEN HUMAN VON WILLEBRAND FACTOR AND COAGULATION FACTOR VIII

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Factor VIII and von Willebrand factor are two plasma proteins essential for effective hemostasis. In vivo, they form a non-covalent complex whose association appears to be metal ion dependent. However, a precise definition of the nature of the molecular forces governing their association remains to be defined, as does their binding affinity. In this paper we have determined the dissociation constant and stoichiometry for Factor VIII binding to immobilized von Willebrand factor. The data demonstrate that these proteins interact saturably and with relatively high affinity. Computer assisted analyses of the Scatchard data favour a two site binding model. The higher affinity site was found to have a K_d of 62 ($\pm$13) x 10^{-12} M while that of the lower affinity site was 380 ($\pm$92) x 10^{-12} M. The density of Factor VIII binding sites (B_{max}) present on von Willebrand factor was 31($\pm$3) pM for the high affinity binding site and 46($\pm$6) pM for the lower site, corresponding to a calculated Factor VIII: von Willebrand factor binding ratio of 1:33 and 1:23, respectively.

Blood coagulation and thrombosis depend on the targeted interaction of a series of plasma proteins with blood platelets, causing the formation of a hemostatic plug at the site of vascular injury. FVIII and vWf are two of the many plasma proteins essential for effective hemostasis. Defects in the FVIII or vWf gene can cause the congenital bleeding disorders of hemophilia A or von Willebrand's disease, respectively. At the molecular level, FVIII functions as a cofactor in the activation of Factor X to Factor Xa. This reaction also requires Factor IXa, calcium and phospholipids, forming the FX activating complex which, in turn, initiates subsequent steps in the coagulation pathway.

FVIII has been purified and structurally characterized by a number of different groups (1-5). In plasma, FVIII is a heterodimer consisting of a series of heavy chains of mass 210-92 kDa linked to a light chain of mass approximately 80 kDa (1-4). Less than 5% of the circulating FVIII exists as a single chain form (5). The cDNA for FVIII has been isolated, sequenced and expressed (for reviews see 6-8). vWf is a large multimeric glycoprotein which functions as an adhesive molecule, mediating platelet vessel wall interactions at the site of vascular injury (9). In addition, vWf plays an essential role as a carrier protein for FVIII; it stabilizes FVIII in plasma (10), and in tissue culture when it is expressed from transfected cells (11). vWf exists in blood as a series of disulfide linked protomers or multimers. Each protomer is a dimer composed of two identical subunits of molecular mass approximately 240 kDa (12) and vWf multimers range in size from 1 - 20 x 10^6 Da. The cDNA for vWf has also been isolated and sequenced by a number of research groups (13-15).

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Abbreviations: **FVIII**, Factor VIII; **vWf**, von Willebrand factor; **TBST**, Tris-buffered saline/Tween; **BSA**, bovine serum albumin; **FPLC**, fast protein liquid chromatography.

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In blood, vWf and FVIII form a non-covalent complex (16). This association appears to be metal ion dependent (17,18) and, based on the plasma concentrations and the molecular weights of the single chain form of these proteins, it can be estimated that vWf is less than 1% saturated with FVIII in vivo. The binding site for FVIII on vWf has been mapped to the vWf N-terminus, between amino acids 1 and 272 (19,20) while the binding site for vWf on FVIII has been localized to the light chain (21) between amino acids 1673 - 1684 (22,23). However, a precise definition of the nature of the molecular forces governing the association of FVIII with vWf remains to be defined, as does their binding affinity.

In this paper we describe the use of a sensitive ELISA, in conjunction with purified human vWf and FVIII, to determine the dissociation constant and stoichiometry for FVIII binding to vWf.

MATERIALS AND METHODS

Preparation of Purified vWf: vWf was purified using a modification of a procedure described previously (24). Cryoprecipitate was prepared from plasma of blood anticoagulated with citrate phosphate dextrose adenine collected from normal donors at our Blood Centre according to methodology outlined elsewhere (25). Routinely, 4 units of cryoprecipitate were reconstituted using Buffer A (0.02 M sodium citrate, 0.15 M NaCl, pH 7.4) containing the protease inhibitor tranexamic acid (KabiVitrum, Sweden) at a final concentration of 20 μg/mL. In order to precipitate contaminating fibrinogen and fibronectin, the resuspended cryoprecipitate (designated Fraction I) was acidified to a pH of 6.4 with 1 N acetic acid, then incubated in a 8°C waterbath. After 2 h the suspension was centrifuged for 10 min x 4000 g at 8°C. The supernatant fraction (containing greater than 80% of the vWf, designated Fraction II) was then adjusted to a pH of 7.4. To remove albumin, Fraction II was chromatographed over Affi-Gel Blue Agarose (Bio-Rad, Richmond, CA) equilibrated with Buffer A. Eluted fractions (monitored by adsorbance at 280 nm, and referred to as Fraction III) containing the peak of eluted vWf activity were pooled and concentrated approximately 15-fold by ultrafiltration (Immersible CX, Millipore). The final step in purification was FPLC (Pharmacia Ltd., Montreal, Que.) using Superose as the gel permeation matrix. The column was pre-equilibrated with Buffer A containing 20 μg/mL tranexamic acid. Protein content of the eluted fractions was measured spectrophotometrically. Fractions with the highest specific activity (ristocetin co-factor activity per mg protein) were pooled and designated as Fraction IV. Throughout fractionation, samples of Fractions I-IV were immediately assayed for vWf and FVIII activities, and additional aliquots were stored at -40°C for later analyses.

Preparation of Purified FVIII: FVIII was fractionated to homogeneity from either heparinized plasma or commercial FVIII concentrates (the latter kindly provided by Dr. M. Mozen, Cutter/Miles Biologicals, Berekely, CA), by a modification of a procedure described in detail elsewhere (5). In brief, it consisted of sequential fractionation by cryoprecipitation, polyethylene glycol precipitation, Affi-Gel Blue Agarose, Aminohexyl Sepharose (Pharmacia Ltd., Montreal, Que.), and immunoaffinity chromatography utilizing a monoclonal antibody to FVIII (Bioscot Laboratories, Edinburgh). For FVIII concentrates derived from citrated plasma, fractionation was initiated at the Aminohexyl Sepharose step.

Electrophoretic Analyses and Immunoblotting: SDS PAGE analysis under reducing conditions was performed using slab gels in the discontinuous buffer system of Laemmli (26) with a stacking gel of 3.5% (w/v) acrylamide and a resolving gel of 6% (w/v) acrylamide. Molecular mass standards (Bio-Rad, Mississauga, Ont.) included in each gel were myosin (200 kDa), beta-galactosidase (116 kDa), phosphorylase a (94 kDa), albumin (68 kDa) and ovalbumin (43 kDa). Silver staining was done using a modification of the method of Switzer et al. (27) as described previously (5). Immunoblotting using polyclonal or monoclonal anti-vWf (where specified) (Behring Diagnostics, Montreal, Que.) or monoclonal anti-FVIII (Synbiotics, San Diego, CA) was carried out as described elsewhere (24,28).

ELISA for Measuring Binding of FVIII to vWf: To coat microtitre plates, 65 ng of purified vWf in 250 uL of Buffer B (0.0285 M sodium barbital, 0.0285 M sodium acetate, 0.116 M NaCl, 20 μg/mL tranexamic acid, pH 7.3) was added to each well of flat bottom microtitre plates (Immulon II, Dynatech) (29). After overnight incubation at 4°C, the wells were aspirated and blocked with buffer C (Buffer B containing 3% (w/v) BSA and 0.05% (w/v) Tween 20) for 1 h at 37°C, washed with TBST (10 mM Tris, 150 mM NaCl, pH 7.4, and 0.05% (w/v) Tween 20) and then aspirated prior to adding the ligand.

FVIII was quantified by a modification of an ELISA method published previously (30). FVIII (0-400 ng/mL) was added according to the experimental design, and the plates incubated for 2 h at 37°C. After washing with TBST, murine monoclonal antibody to FVIII was added, and incubation continued for 1 h. The epitope for this antibody mapped to the carboxyl terminal region of FVIII (C2 domain) and did not inhibit FVIII binding to vWf (P. Ganz, unpublished observations). The wells were then washed with TBST and alkaline phosphatase conjugated goat anti-mouse IgG (Promega Biotech,

Madison, WI) was added for 30 min followed by washing. Substrate solution containing para-nitrophenylphosphate was prepared and added according to the manufacturer's instructions (Protoblot System, Promega Biotech, Madison, WI). The reaction was developed for 18 min before the addition of 3 N NaOH, whereupon the intensity of the colour reaction was measured spectrophotometrically at 405 nm (Titertek Multiscan MC, Flow Laboratories). A standard curve was constructed with each assay using quantities of FVIII from 0 to 400 ng/well. The OD_{405} values from the binding experiments were converted to ng of FVIII bound from the standard FVIII curve, and the amount of FVIII bound for each concentration determined as the mean of triplicates. The amount of non-specific FVIII binding was assessed from wells in which BSA was substituted for vWf. This value was then subtracted from total binding to yield values for the specific binding of FVIII. The data were analysed according to the method of Scatchard (31) using the computerized methodology embodied in the Ligand program (32).

Assay of vWf Activity: A modification of the method of Macfarlane et al. (33) was employed to quantitate ristocetin cofactor activity. Formalin fixed and washed platelets were incubated with the test sample and allowed to aggregate (Chrono-Log, Lumi Aggro-meter) in the presence of ristocetin (Lenau, Denmark). The slope of each aggregation curve was calculated and a standard curve was produced using dilutions of commercial plasma prepared from a pool of 20 normal individuals.

Assay of FVIII Procoagulant Activity: FVIII procoagulant activity was measured by the one-stage clotting assay, a modification of the activated partial thromboplastin time (34). Samples were tested in duplicate using lyophilized reference plasma as a standard (Pacific Haemostasis, Bakersfield, CA). Clot times were determined on a dual channel Coag-A-Mate (General Diagnostics, Morris Plains, NJ). Immunological assays of FVIII were carried out as described previously (30).

Protein Determinations: Protein concentrations were quantified by the method of Bradford (35) using BSA as a reference standard.

RESULTS

vWf was purified approximately 11,000-fold over starting material, with a specific activity of 160 U/mg (Figure 1A). This level of purity is within 10% of the specific acitivity reported by Chopek et al. (12) for purification of vWf from commercial concentrates using a different purification scheme. Analyses of the purified vWf (Fraction IV) by SDS PAGE followed by silver staining revealed a major band of mass 240 kDa corresponding to the major subunit of vWf (12) (Fig 1A, INSERT Lane 1). To confirm that the 240 kDa polypeptide corresponded to vWf, immunoblotting was carried out with anti-vWf as a probe. Fig 1A (INSERT Lane 2) shows the reactivity of the 240 kDa band with this antibody confirming that it represents vWf. This material was used in the subsequent binding experiments.

FVIII was purified approximately 250,000-fold over plasma with a final specific activity of 5200 U/mg as reported earlier (5) (Figure 1B). Analyses of the purified FVIII by SDS PAGE followed by silver staining (Fig 1B, INSERT Lane 3) revealed the typical mixture of heavy (mass 210 kDa to 92 kDa) and light (doublet at 80 kDa) chain pattern characteristic of FVIII (1-3,21). Western blotting using an antibody specific for the light chain of FVIII identified the 80 kDa doublet as FVIII (Figure 1B, INSERT Lane 4).

Using a quantitative ELISA, studies were carried out to define the dissociation constant of binding between vWf and FVIII, as well as to determine the amount of FVIII bound to vWf under saturating conditions. As shown in Figure 2, when the concentration of FVIII added to vWf coated wells was increased from 0 to 320 ng/mL, there was a progressive uptake of FVIII by the immobilized vWf. Above a concentration of 320 ng/mL, no further uptake was observed, indicating saturable binding.

Data from Figure 2 were analysed according to Scatchard (31) as shown in Figure 3. The plots that were obtained were curvilinear, suggesting that the data would best fit a two-site binding model. The binding parameters calculated from the data are summarized in TABLE 1. FVIII is shown to interact with two sites on vWf, a high affinity site with a dissociation constant (K_d) of 62 ($\pm$13) x 10^{-12}, and a lower affinity site with a calculated K_d of 380 ($\pm$92) x 10^{-12} (TABLE 1). The difference in binding affinities between the higher and lower affinity site is approximately six-fold.

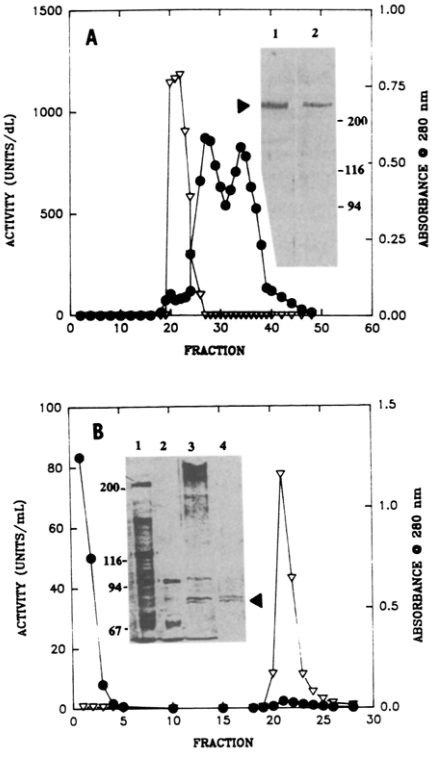
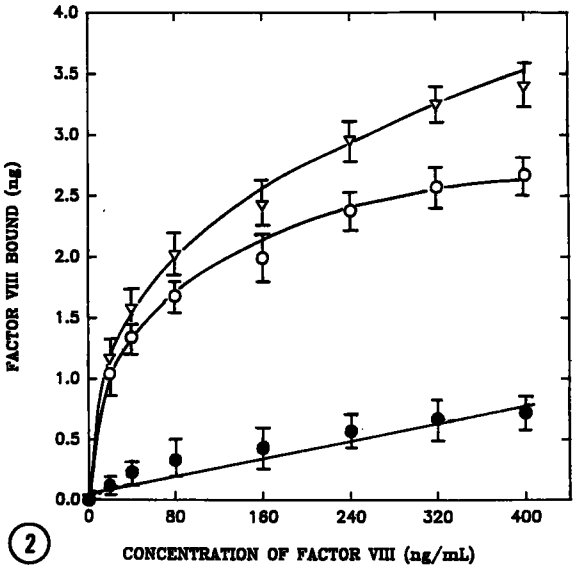


FIG. 1. Preparation of purified vWf and FVIII. (A) FPLC of vWf on Superose. Approximately 10 mL of vWf (Fraction IV) was loaded onto 180 mL of Superose prewashed with buffer A. Fractions were assayed for absorbance at 280 nm (●) and ristocetin cofactor activity (▽) (U/dL). (INSERT) SDS PAGE and immunoblotting of the purified vWf. Lane 1, silver stained 6% polyacrylamide SDS gel run under reducing conditions. Arrows indicate the 240 kDa vWf subunit; positions of molecular weight markers in kDa are indicated. Lane 2, immunoblot of purified vWf showing the major 240 kDa subunit of vWf. (B) Immunoaffinity chromatography of FVIII. Approximately 700 Units of FVIII were loaded onto a 25 mL column of Affi-Gel 10 containing monoclonal anti-FVIII antibody. Following a high salt wash (1M NaCl, 4 mM $CaCl_2$ in 20 mM Tris-HCl buffer, pH 7.4) the protein was eluted with 50% ethylene glycol in this same buffer. Eluted fractions (5 mL) were monitored for absorbance (●) at 280 nm and assayed for procoagulant activity (▽). (INSERT) SDS PAGE and immunoblotting of the purified FVIII preparation. Lanes 1, 2, silver stained gels of high and low molecular weight markers. Lane 3, silver stained gel of purified FVIII showing mixture of heavy and light chains (210 kDa-80 kDa). Lane 4, immunoblot of material in Lane 3, developed using anti-FVIII light chain antibody; arrow indicates FVIII light chain (80 kDa).

The total density of FVIII binding sites (B_{max}) present on the immobilized vWf is also given in TABLE 1. For the high affinity binding sites the receptor density was estimated as 31 (±3) pM, whereas the density of the lower affinity binding sites was calculated as 46 (±6) pM. These values correspond to a calculated binding ratio of 1 molecule of FVIII to 33 molecules of vWf, and 1 molecule of FVIII to 23 molecules of vWf, for the high and lower affinity binding sites, respectively.

DISCUSSION

Using purified preparations of human vWf and FVIII in conjunction with a sensitive ELISA, we have determined the stoichiometry and dissociation constant for FVIII binding to vWf. FVIII and vWf interact with relatively high affinity and exhibit characteristics of two site binding. A dissociation constant of 62 (±13) x 10^{-12} M was observed for one binding site and a K_d of 380 (±92) x 10^{-12} M was determined for a second, lower affinity site. Two research groups have identified the location for



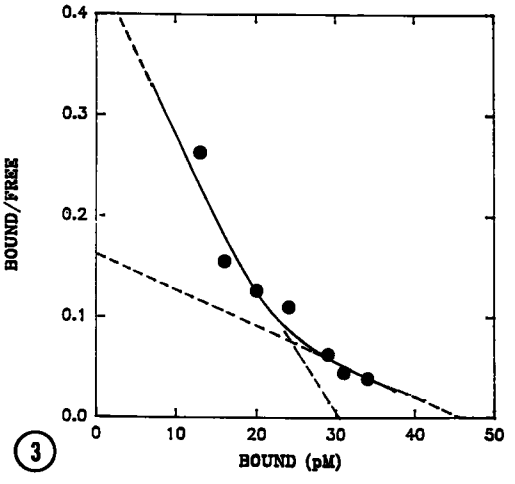


FIG. 2. Concentration dependency of binding of FVIII to vWf. Microtitre plates were coated with 65 ng of purified vWf. Purified FVIII (250 uL) at the concentrations shown were then added. After a 2 h incubation the amount of FVIII bound was quantified as described under Materials and Methods. (▽) FVIII total bound; (o) FVIII specifically bound; (●) level of non-specific binding. Values shown represent means (±SEM) of 12 separate experiments.

FIG. 3. Scatchard plots of the binding of FVIII to vWf. Data shown in Figure 2 were plotted as described by Scatchard (31) and analyzed according to the Ligand program of Munson and Rodbard (32). Dashed lines indicate the projected linear components of the binding isotherms.

binding of FVIII on the vWf molecule. Takahashi et al. (20) have reported that FVIII binds in a dose-dependent manner to an immobilized S. aureus V8 protease cleavage product (SpIII) derived from the N-terminal portion of vWf. No binding to cleavage products recovered from the central (SpII) or C-terminal (SpI) portions of vWf was observed. Additionally, monoclonal antibodies which map to the SpIII fragment also inhibited the binding of FVIII. These investigators thus concluded that the FVIII binding site encompasses vWf residues 1 to 910. In another study by Foster et al. (19), the FVIII binding domain of vWf was mapped between vWf residues 1 and 272 (an approximately 32-34 kDa fragment). These data, with the results of the present study, therefore indicate that FVIII could bind to at least two sites within this 32-34 kDa N-terminal fragment of vWf, albeit with differing affinities.

Identification of the residues within the N-terminal region of vWf that contribute directly to the binding of FVIII has not yet been achieved. However, it is possible that there are von Willebrand's

TABLE 1

BINDING PARAMETERS DERIVED FROM SCATCHARD ANALYSES FOR THE ASSOCIATION OF PURIFIED FVIII WITH IMMOBILIZED vWf

LIGAND	IMMOBILIZED PROTEIN	BINDING SITE	K_d VALUE*	B_{max} pM	RATIO FVIII:vWf
FVIII	vWf	HIGH AFFINITY	62 (±13) x 10^{-12}	31 (±10)	1:33
FVIII	vWf	LOW AFFINITY	380 (±92) x 10^{-12}	46 (±13)	1:23

*Values shown represent ±SEM, n=12.

disease variants that exhibit altered vWf binding of FVIII. In this regard, Nishino et al. (36) have described two patients with a new variant of von Willebrand's disease. The vWf from these propositi exhibit defective binding for FVIII, structural abnormalities when the multimer pattern of the protein is analysed. Interestingly, even though the binding of FVIII was significantly reduced, it was not eliminated. This may reflect a vWf alteration in only one of the two FVIII binding sites.

FVIII and vWf are known to circulate as a non-covalent complex in vivo (16). This association appears to be metal ion dependent (17,18,37). Based on the plasma concentrations and the molecular weights of these proteins (FVIII, plasma concentration of approximately 100 ng/mL and mass of 265 kDa; vWf, plasma concentration of approximately 10 μg/mL and a mass of approximately 240 kDa), it can be estimated that vWf in blood is not more than 1% saturated with FVIII. Studies carried out by Zucker et al. (38) in which partially purified preparations of vWf and FVIII were mixed and formation of the resultant complexes analysed by gel filtration showed that 12 times the amount of plasma FVIII could be loaded onto vWf. Hamer et al. (21) determined that one FVIII molecule was bound for every fourth molecule of vWf. In a porcine system where FVIII was provided in excess concentration in vitro, the stoichiometry of the porcine vWf-FVIII complex has been determined as 1.2 vWf monomers per FVIII molecule under saturating conditions (39). The experiments reported here indicate a stoichiometry of binding of one molecule of FVIII per thirty-three molecules of vWf (high affinity site) and one molecule of FVIII for every twenty-three molecules of vWf (low affinity site). The deviation observed in vitro from the predicted theoretical stoichiometry of 1:1 is likely a consequence of some denaturation of the vWf molecules during purification.

The relatively high affinity association between FVIII and vWf revealed by this study has several important implications for understanding how FVIII interacts with platelet membranes. At sites of vascular injury, vWf is known to function as an adhesive molecule contributing to the adhesion of platelets to the exposed subendothelium. vWf binds to the platelet glycoprotein Ib (9,40,41) and may also bind to glycoprotein IIb-IIIa (9,42). Following the binding of the vWf-FVIII complex to glycoprotein Ib, it is assumed that FVIII is dissociated from vWf and subsequently binds tightly to the platelet surface where it serves as the cofactor in the Factor IXa catalysed activation of Factor X. The mechanism whereby FVIII is dissociated from vWf and transposed to the platelet membrane is unknown. Given that the dissociation constant of FVIII binding to vWf reported here is in the pM range, it is unlikely that FVIII can be competed from vWf by specific FVIII platelet receptors. Dissociation constants in the nM range have been reported for FVIII binding to its platelet binding sites (43,44). Moreover, the K_d for binding of FVIII to synthetic phosphatidylserine containing phospholipids has been estimated as 2-4 nM (45) indicating that negatively charged phospholipids exposed on the surface of platelets following activation likely do not form a binding site of sufficiently high affinity to compete away FVIII from vWf. In sum, on the basis of the high affinity association between FVIII and vWf described here, it seems unlikely that either phospholipid or proteinaceous receptor sites are of sufficiently high affinity to dissociate the FVIII/vWf complex, suggesting that another mechanism exists to accomplish this.

It is known that both thrombin and Factor Xa cleave FVIII between amino acid residues 1689 and 1690 within the 80 kDa light chain, to generate the 72 kDa light chain of FVIIIa (21). This causes the release of thrombin activated FVIII from vWf (21) and therefore may explain how FVIII is delivered to the platelet membrane surface. However, this scheme requires that thrombin be available as soon as the FVIII/vWf complex binds to platelets. An alternative mechanism is that release of calcium from platelets, concomitant with platelet activation, triggers the dissociation of FVIII from the glycoprotein Ib associated vWf/FVIII complex. Clearly, more studies are required to resolve these different possibilities.

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