The Acidic Region of the Factor VIII Light Chain and the C2 Domain Together Form the High Affinity Binding Site for von Willebrand Factor

(Received for publication, November 20, 1996, and in revised form, May 14, 1997)

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A binding site for von Willebrand factor (vWF) was previously localized to the carboxyl terminus of the C2 domain of the light chain (LCh) of factor VIII (fVIII). The acidic region of the LCh, residues 1649–1689, also controls fVIII-vWF binding by an unknown mechanism. Although anti-acidic region monoclonal antibodies prevent formation of the fVIII-vWF complex, the direct involvement of the acidic region in this binding has not been demonstrated. By limited proteolysis of LCh with Staphylococcus aureus V8 protease, we prepared 14- and 63-kDa LCh fragments, which begin with fVIII residues 1672 and 1795, respectively. Using surface plasmon resonance to measure binding interactions, we demonstrated that the 14-kDa fragment binds to vWF, but its affinity for vWF ($K_d = 72$ nM) was 19-fold lower than that of LCh. This was not due to an altered conformation of the acidic region within the 14-kDa fragment, since its affinity for an anti-acidic region monoclonal antibody was similar to that of LCh. All LCh derivatives lacking the acidic region (thrombin-cleaved LCh, recombinant C2, and 63-kDa fragment) had also greatly reduced affinities for vWF ($K_d = 564–660$ nM) compared with LCh ($K_d = 3.8$ nM). In addition, the similar affinities of these derivatives for vWF indicated that apart from its acidic region, the LCh contains no vWF binding site other than the one within C2. The reduced affinities of the LCh derivatives lacking the acidic region for monoclonal antibody NMC-VIII/5 (epitope, C2 residues 2170–2327) indicated that removal of the acidic region leads to a conformational change within C2. This change is likely to affect the conformation of the vWF binding site in C2, which overlaps the epitope of NMC-VIII/5; therefore, the acidic region also appears to be required to maintain the optimal conformation of this vWF binding site. Our results demonstrate that the acidic region and the C2 domain are both directly involved in forming a high affinity binding site for vWF.

The plasma glycoprotein factor VIII (fVIII)† functions as a cofactor for factor IXa in the factor X activation enzyme complex of the intrinsic pathway of blood coagulation (1). fVIII internal protein sequence homology has led to the designation of six domains arranged in the order A1-A2-B-A3-C1-C2 (Ref. 2; Fig. 1). The heavy chain (HCh) of fVIII consists of the A1, A2, and B domains, whereas the light chain (LCh) consists of the A3, C1, and C2 domains. Three regions rich in acidic amino acids are located at the carboxyl termini of the A1 and A2 domains and the amino terminus of the A3 domain.

Maintenance of a normal fVIII level in the circulation is dependent on its complex formation with von Willebrand factor (vWF) since patients with severe von Willebrand’s disease, who have a complete deletion of the vWF gene or mutations which reduce binding between fVIII and vWF, have a secondary deficiency of fVIII. When fVIII is bound to vWF, a stable association of its HCh and LCh is maintained (3), and fVIII is prevented from binding to phospholipid vesicles (4), platelets (5), or factor IXa, functions required for its procoagulant activity. In addition, vWF protects fVIII from activation by activated factor X (6) and from protein C-catalyzed inactivation (4). fVIII binds to vWF through the LCh (7, 8). The reported binding stoichiometry is 1 fVIII molecule/vWF monomer (9, 10).

Thrombin, the principal physiological activator of fVIII, cleaves the protein at Arg$^{172}$ and Arg$^{740}$ in the HCh and at Arg$^{1689}$ in the LCh (11). Activated fVIII (fVIIIa) is a heterodimer of 50-, 43-, and 73-kDa subunits, all of which are required for procoagulant activity (12). Cleavage of the LCh at Arg$^{1689}$, which releases the acidic region (fVIII residues 1649–1689), is responsible for dissociation of fVIIIa from vWF (8, 13). The importance of the LCh acidic region for fVIII binding to vWF was suggested by the observations that several anti-acidic region monoclonal antibodies (mAbs) with epitopes within residues 1670–1689 (14–17) inhibit fVIII binding to vWF, as does complete deletion of the acidic region (18). In contrast, deletion of the fVIII B domain and part of the acidic region (1649–1669) did not abolish vWF binding (18), suggesting that the acidic region residues 1669–1689 are critical for fVIII-vWF binding. Mutants of fVIII with partial or complete deletions of the acidic region have normal procoagulant activity (18), which suggests that the loss of this region has no effect other than the elimination of vWF binding. The presence of post-translationally sulfated Tyr$^{1680}$ was shown to be essential for vWF binding. However, synthetic peptide 1673–1689 failed to inhibit fVIII binding to vWF, regardless of whether Tyr$^{1680}$ was sulfated (18). Thus, the exact function of the acidic region is not known, since its direct binding to vWF has not been demonstrated.

We demonstrated that a glutathione S-transferase-C2 fusion protein binds to immobilized vWF in a dose-dependent, saturable fashion (19), which indicates that the C2 domain also com-
of these controls were \( \leq 10\% \) of the maximal signal, and they were subtracted from the values for all other samples.

**Protein Purification**—Plasma FVIII was purified from therapeutic concentrates of Method M, American Red Cross (30). LCh and HCh were purified as described previously (12, 21). Residual HCh was removed from the LCh preparation by its precipitation by its purified mAb 8860 column. The purity of the LCh was confirmed by SDS-PAGE, where it migrates as a single band of 80 kDa (Fig. 2, lane 1). Residual LCh present in the HCh preparation was removed by passage over an immobilized mAb ESH8 column equilibrated with 20 mM Tris, pH 7.4, 0.15 M NaCl (TBS), 5 mM CaCl\(_2\). The final HCh preparation (2650 nM) contained \( \leq 0.025 \) nM LCh as tested by ELISA 1. A3-C1-C2 was further purified by cleavage of LCh with thrombin (31) and purification by ion exchange chromatography on a Resource S column (Pharmacia Biotech Inc.) (32). Traces of uncleaved LCh were removed by incubation for 18 h at 4 °C in TBS, 5 mM CaCl\(_2\), 0.01% Tween 20 with the anti-acidic region mAb C4 immobilized on CNBr-activated Sepharose 2B resin at 1.4 mg/ml. The final A3-C1-C2 preparation (3700 nM) contained \( < 0.03 \) nM LCh, as measured by ELISA 2. The purity of A3-C1-C2 was also confirmed by SDS-PAGE, where it migrates as a single band with a molecular mass of 73 kDa (Fig. 2, lane 2). The FVIII HCh/A3-C1-C2 heterodimer was prepared from HCh and A3-C1-C2 and purified by ion exchange chromatography on a Resource S column (31). Its activity was determined in the one-stage clotting assay (33) to be 3300 units/mg, which is similar to a previously reported value (31). The recombinant C2 domain was produced as a soluble, secreted protein in Spodoptera frugiperda SF9 insect cells (34), and it was purified by gel filtration and ion-exchange chromatography (21). vWF was purified from cryoprecipitate (Cutter Biological) (21). The final vWF preparation (0.68 mg/ml) contained \( < 0.2 \) \( \mu \)g/ml FVIII, as determined by an immunoradiometric FVIII antigen assay (35).

**Radiolabeling of FVIII**—Prior to iodination FVIII was dialyzed into 0.2 M sodium acetate, 5 mM calcium nitrate, pH 6.8 (iodination buffer). Five \( \mu \)g of FVIII in 30 \( \mu \)l of iodination buffer were added to 5 \( \mu \)l of lactoperoxidase beads (50% activity, Worthington Biochemical Corp.), 5 \( \mu \)l of Na\(^{251}\)I (100 mCi/ml, Amersham), and 5 \( \mu \)l of 0.03% H\(_2\)O\(_2\) (Mallinkrodt) and incubated for 3 min. Free Na\(^{251}\)I was removed by chromatography on a PD10 column (Pharmacia) in 0.1 M MES, 0.15 M NaCl, 5 mM CaCl\(_2\), pH 6.3. The specific radioactivity of FVIII was 4 \( \mu \)Ci/\( \mu \)g of protein. The activity of \( ^{125}\)I-FVIII determined in the one-stage clotting assay (3600 units/\( \mu \)g) was similar to that of unlabeled FVIII.

**Analysis of 14-kDa Fragment and LCh Cleavage by SDS-PAGE**—The kinetics of 14-kDa fragment and LCh thrombin cleavage at Arg\(^{1590}\) was analyzed by electrophoresis in 8–25% SDS-PAGE, followed by staining with Coomassie blue. Protein bands were quantitated by an HP ScannerJet IICx scanner (Hewlett Packard) and the NIH Image computer program. Cleavage of LCh or 14-kDa fragment at Arg\(^{1590}\) was determined as the ratio of cleaved product migrating as 73 or 11.5-kDa bands, respectively, to total protein in the lane.

**Protein-Protein Binding Using Biosensor Technology**—The kinetics of protein-protein interaction were determined by surface plasmon res-
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The LCh of fVIII binds to vWf with lower affinity than fVIII. To determine whether LCh is solely responsible for the high affinity fVIII interaction with vWf, we compared kinetics of fVIII and LCh binding to and dissociation from vWf immobilized on a biosensor cuvette using an IAsys biosensor instrument. The detection of bound protein is based on the surface plasmon resonance phenomenon, which measures the change in the refractive index due to association of a fluid phase ligand with an immobilized ligand or due to dissociation of the formed complex (36) by generating a signal of 165 Arc s/l mg of protein bound/mm² of the biosensor cuvette.

Representative sensograms in Fig. 3A show the resonance response reflecting time course of fVIII or LCh association interaction with immobilized vWf. Similar binding to a control cuvette lacking vWf by maximal concentrations of fVIII or LCh gave resonance signals ≤1% of those in the presence of vWf (Fig. 3A), indicating the absence of nonspecific interactions with the cuvette.
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The $k_{on}$ and $k_{off}$ values were derived from dissociation curves for fVIII and LCh (Fig. 3B) using Equation 1 describing single exponential dissociation to the base line (see “Experimental Procedures”). The values of second order association rate constants ($k_{on}$), shown in Table I, were determined from the best fit of association kinetics data to Equation 3 describing single phase association (see “Experimental Procedures”). Equilibrium binding was achieved for fVIII and LCh after 1 and 3 min (Fig. 3A), respectively. Since the concentrations of fVIII or LCh were >10 times the corresponding $K_d$ values for their interaction with vWf (Table I), the amounts of fVIII and LCh bound to vWf at equilibrium (3.8 fmol/mm² and 3.62 fmol/mm², respectively) represent the maximal binding capacity of immobilized vWf. The binding capacity of immobilized vWf, which is only 20% of the concentration of immobilized vWf (18.5 fmol/mm²). This is a typical percent for dextran-immobilized protein molecules accessible for ligand binding (39).

The $k_{off}$ for the fVIII-vWf complex was 14.8-fold lower than that for the LCh-vWf complex, whereas the $k_{on}$ values for these complexes were similar. The $K_d$ for fVIII-vWf binding in our experiments (0.4 nm) was similar to that of 0.2–0.46 nm determined in previous studies (5, 10, 17, 40) and 9.5-fold higher than that of LCh (3.8 nm), suggesting that HCh is involved in fVIII-vWf binding. Since HCh up to 2 µM did not bind vWf (data not shown), its participation in fVIII binding to vWf is indirect.

**Purification and Characterization of LCh Proteolytic Fragments**—The V8 protease from *S. aureus* cleaves LCh into two fragments with molecular masses of 14 and 63 kDa, as determined by SDS-PAGE (Fig. 2, lane 3), which remain associated and are designated below as the 14-kDa/63-kDa complex. Since these fragments contain either the acidic region or C2, we purified them further for studies of the role of each region in vWf binding. Isolation of the individual fragments required denaturing conditions (9 M urea) and refolding as described under “Experimental Procedures.” The refolded 14- and 63-kDa fragments exhibited fluorescent spectra with $\lambda_{max}$ at 332 and 335 nm, respectively, which indicate that Trp residues are partially buried, and therefore it is likely that fragments had regained a compact structure. Size exclusion chromatography on a Superose 12 HR (Pharmacia) column revealed that the fragments eluted as expected for polypeptides of 14 and 63 kDa, indicating the absence of intermolecular aggregates (data not shown).

Amino-terminal sequencing (10 cycles) of the purified 14- and 63-kDa fragments demonstrated that they begin with residues 1672 and 1795, respectively (data not shown), indicating that *S. aureus* V8 protease cleaves LCh after Glu²⁶⁷¹ and Glu¹⁷⁹⁴ (Fig. 1). The molecular masses of the fVIII sequences 1672–1794 and 1953–2323 calculated from the deduced protein sequence of fVIII (41) are 14.4 and 63.5 kDa, respectively. Although the carboxyl termini of the fragments were not determined, the similarity of the calculated masses for the proposed fVIII sequences to that determined for the fragments by SDS-PAGE analysis and silver staining (Fig. 2, lanes 4 and 5), suggests that the termini (Fig. 1) are close to the expected residues 1794 and 2332.

If the isolated 14- and 63-kDa fragments are in a native conformation, it would be expected that the 14-kDa/63-kDa heterodimer can be reconstituted from the isolated fragments. As shown in Fig. 4C, the equimolar mixture of 14- and 63-kDa fragments elutes as a single peak by size-exclusion chromatography with a molecular mass >66 kDa. Analysis of the material from the peak by SDS-PAGE confirmed the presence of both 14- and 63-kDa fragments (data not shown). The binding between 14- and 63-kDa fragments is apparently of high affinity, since a very little material contained in the peak corresponding to 14-kDa fragment in the equimolar mixture of 14- and 63-kDa fragments. Increasing proportion of the 63-kDa fragment in the mixture caused a steady reduction of the area of the peak corresponding to 14-kDa fragment while increasing that of the complex. A plot of the peak area of the 14-kDa fragments versus the molar ratio of the 63-kDa and 14-kDa fragments in the mixture revealed the formation of an equimolar complex (Fig. 4C, inset).

**LCh Derivatives Lacking the Acidic Region Bind to vWf with the Low Affinity of C2**—If no LCh regions other than C2 and the acidic region are involved in vWf binding, affinities of A3-C1-C2 and HCh/A3-C1-C2 heterodimer will be similar. As seen from the kinetic curves measured using the biosensor technique (Fig. 5), association of A3-C1-C2, C2, or 63-kDa fragment with vWf approached equilibrium within 1.5 min. The observed association and dissociation kinetics were optimally described by assuming one single class of vWf binding sites for each of the above ligands. The $k_{on}$ values for A3-C1-C2, C2, and 63-kDa fragment derived from the association curves (Fig. 5A) using Equation 3 (see “Experimental Procedures”) were $6.4 \times 10^4$, $6.2 \times 10^4$, and $5.0 \times 10^4$ M⁻¹ s⁻¹ (Table I), respectively. The similar $K_d$ values for each ligand demonstrate that removal of the LCh acidic region leads to a similar reduction in vWf affinity for the remaining part of the LCh.

Since <$0.003$ nm LCh was present in 400 nm A3-C1-C2 used in these experiments (see “Experimental Procedures”), we measured binding of 0.003 nm LCh to vWf as a control, but none was observed during 2 min (data not shown). Preincubation (30 min, 37 °C) of 400 nm A3-C1-C2 with 1200 nm anti-acidic region mAb NMC-VIII/10 (which inhibits fVIII-vWf binding; Ref. 15) had no effect on its binding to vWf (data not shown), which also indicates that A3-C1-C2 binding to vWf was not due to its contamination with residual acidic region in uncleaved LCh. In contrast, preincubation of A3-C1-C2 with anti-C2 mAb NMC-VIII/6 resulted in $\approx 95\%$ reduction of its binding to vWf (Fig. 5), which demonstrates that A3-C1-C2 binding to vWf is specific and occurs only through the C2 domain.

To determine if HCh has any effect on A3-C1-C2 binding to vWf, the affinities of A3-C1-C2 and HCh/A3-C1-C2 het-
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Figure 4. Association of 14- and 63-kDa fragments. Size-exclusion chromatography of 4 μM 14-kDa fragment alone (A) and mixed with 2 μM (B) and 4 μM (C) amounts of 63-kDa fragment. The volume of the sample loaded on the column was 0.25 ml in all experiments. The inset in panel C plots the decrease in area of the 14-kDa peak versus the molar ratio of the 63-kDa fragment to 14-kDa fragment. The column was calibrated with standard proteins of molecular masses of 66, 29, 12.4, and 6.5 kDa, respectively.

Figure 5. The kinetics of binding of FVIII derivatives lacking the acidic region and recombinant C2 to vWF. Association of 400 nM each A3-C1-C2 (●), 63-kDa fragment (○), C2 (▲), and HCh/A3-C1-C2 (□) with immobilized vWF (5 ng/mm²) (A) and dissociation of the corresponding complexes (B) was measured, and fitted curves were obtained as in Fig. 3. A3-C1-C2 (400 nM) (○) was preincubated for 30 min at 37 °C with mAb NMC-VIII/5 (800 nM) before addition to immobilized vWF.

Figure 6. Determination of the kinetic parameters for 14-kDa fragment binding to vWF. vWF was immobilized at 10 ng/mm². The association (A) and corresponding dissociation (B) kinetic data were obtained at 50 nM (●), 200 nM (○), and 1000 nM (□) of 14-kDa fragment. The fitted curves (solid lines) were obtained as in Fig. 3. In the experiment shown by ▲, binding of thrombin-cleaved 14-kDa fragment (1000 nM) was measured. In the experiment shown by ○, binding of the 14-kDa fragment (1000 nM) to a cuvette without vWF was measured.

The kₐ values for all FVIII derivatives lacking the acidic region were similar within 10–13-fold lower than that for FVIII association with vWF (Table I). The kₐ values for the above ligands were also similar and 143–170-fold greater than that for FVIII. The half-lives for dissociation of the ligand-vWF complexes, calculated as ln2/kₐ, were 17–21 s. These data suggest that removal of the acidic region results both in a slower rate of association of the ligands with vWF and in a much higher rate of dissociation of the formed complexes. The similar kₐ values for A3-C1-C2, 63-kDa fragment, C2, and HCh/A3-C1-C2 binding to vWF indicate the C2 domain contains the only vWF binding site within FVIII derivatives lacking the acidic region.

The interaction of the 14-kDa fragment with immobilized vWF was tested at varying concentrations. The representative association and dissociation kinetics data are shown in Fig. 6 for three different concentrations of 14-kDa fragment. The kₐ and kₐ values (Table I) are the mean ± standard deviation of kₐ and kₐ derived from the three association and dissociation sets of kinetic data (Fig. 6), using Equations 1 and 3, respectively (see "Experimental Procedures"). The values of kₐ and kₐ derived from separate curves were similar within 6% variation and did not depend on the concentration of the 14-kDa fragment. To determine if the kₐ value calculated as the kₐ/kₐ ratio is correct, we calculated the values of equilibrium binding corresponding to 50 and 200 nM of 14-kDa fragment from the equation $B = B_{max}F(K_D + F)$, where $B$ is the equilibrium binding, $F$ is the concentration of unbound ligand, and $B_{max}$ is the maximal binding capacity of immobilized vWF achieved at the concentration of 14-kDa fragment $K_D$. The equilibrium binding values corresponding to 50, 200, and 1000 nM of 14-kDa fragment were 8.7, 17.1, and 25.8 Arc s, respectively. These binding values correspond to 0.054, 0.108, and 0.158 ng of bound protein/mm² of the cuvette surface. Since the amounts of 14-kDa fragment bound to vWF immobilized on 1.8-mm² cuvette surface were 0.0968, 0.187, and 0.284 ng, corresponding to 50, 200, and 1000 nm of added 14-kDa fragment are much less than the amounts of added fragment (140, 560, and 2800 ng, respectively), the concentration of free ligand is similar to the concentration of added ligand. Assuming that $B_{max}$ is achieved at 1000 nm of added 14-kDa fragment, the equilibrium binding values achieved at 50 and 200 nm 14-kDa fragment, calculated from the above equation are 0.055 and 0.11 ng/mm², respectively, and similar to the experimentally observed values, 0.054 and 0.108 ng/mm².

It was demonstrated in the above experiments that the LCh derivatives lacking the acidic region bind to vWF only via the C2
binding site. Therefore, it is expected that thrombin cleavage of the 14-kDa fragment at Arg<sup>1689</sup> completely abolishes its binding to vWF. The kinetics of cleavage of the 14-kDa fragment (1000 nM) or LCh (1000 nM) by 100 nM thrombin (see analysis under “Experimental Procedures”) were similar (data not shown). The >95% conversion of the 14-kDa fragment into the 11.5-kDa product was achieved after 30 min at 25 °C. The 11.5-kDa fragment (1000 nM) did not bind to immobilized vWF (Fig. 6), indicating that the acidic region binding site is destroyed by the cleavage. In control experiments, no binding of 14-kDa fragment to a biosensor cuvette without vWF was observed (Fig. 6). The 14-kDa fragment (1000 nM) also did not bind to immobilized anti-C2 mAb NMC-VIII/5 (data not shown), which indicates that it was not contaminated with 63-kDa fragment or LCh.

LCh and 14-kDa/63-kDa Complex Bind Similarly to vWF—We have shown that the individual 14- and 63-kDa fragments bind to vWF with low affinity. To determine if this is due to the separation of the fragments from each other or to the proteolytic cleavages by <i>S. aureus</i> V8 protease at positions 1672 and 1794 within the LCh, we also compared affinities of LCh and 14-kDa/63-kDa complex for vWF. The kinetic curves for LCh and 14-kDa/63-kDa interaction with vWF (Fig. 7) demonstrated that these ligands bind similarly to vWF. The resonance signal produced by 14-kDa/63-kDa binding to vWF was approximately 5% lower than that for LCh, as expected due to the 4% lower molecular mass of the 14-kDa/63-kDa complex (77 kDa) compared with the LCh (80 kDa). The <i>k</i><sub>on</sub> and <i>k</i><sub>off</sub> values derived for 14-kDa/63-kDa complex and LCh from dissociation and association kinetic data using Equations 1 and 3, respectively, are similar (Table II). In a control experiment, the complex did not bind to a biosensor cuvette without immobilized vWF (Fig. 7). These results demonstrate that proteolytic cleavage within the LCh does not by itself lead to a lower affinity for vWF.

To test if the reconstituted 14-kDa/63-kDa complex has the same vWF binding properties as the original complex, its binding to immobilized vWF was measured as above. Binding of the both complexes was identical (data not shown), indicating that irreversible changes did not occur in the 14- and 63-kDa fragments as a result of their purification under denaturing conditions.

**Determination of <i>v</i>III, LCh, 14-kDa Fragment, and C2 Affinity for vWF in Fluid Phase**—In the above experiments vWF was covalently immobilized to a dextran-coated biosensor cuvette through amino groups. Since this procedure may potentially alter its binding properties, it is possible that the kinetic parameters derived from biosensor kinetic measurements may show deviations from those determined in a fluid phase assay. In order examine this possibility, binding between <sup>125</sup>I-labeled vVIII and vWF was competed by unlabelled fVIII or its fragments in the fluid phase (Fig. 8). The <i>K</i><sub>v</sub> values for fVIII-vWF binding calculated from the best fit of the <sup>125</sup>I-fVIII homologous displacement by unlabelled fVIII was 0.38 ± 0.04 nM. The values of inhibition constants (<i>K</i><sub>i</sub>) for LCh, 14-kDa fragment and C2 domain calculated from heterologous displacement data using the LIGAND program were 4.1 ± 0.6 nM, 84 ± 16 nM, and 470 ± 94 nM, respectively. Since the heterologous displacement experiments were performed using <sup>125</sup>I-fVIII concentration 10 times below <i>K</i><sub>v</sub> for fVIII-vWF binding, it would be expected that <i>K</i><sub>v</sub> values determined for fVIII derivatives are similar to <i>K</i><sub>v</sub> values for their direct binding to vWF. The similarity between the <i>K</i><sub>v</sub> values derived from the fluid phase equilibrium binding data to the <i>K</i><sub>v</sub> values (Table I) determined as the ratios of <i>k</i><sub>off</sub> and <i>k</i><sub>on</sub> kinetic rate constants derived from the association and dissociation biosensor measurements indicate that binding properties of immobilized vWF are similar to that in solution. In addition, thrombin-cleaved 14-kDa fragment was not inhibitory in the above assay (Fig. 8), which is also consistent with lack of its binding to immobilized vWF in biosensor experiments (Fig. 6).

**Dissociation of the 14-kDa/63-kDa Complex Leads to a Conformational Change within the C2 Domain but Not within the Acidic Region**—The high affinity of the 14-kDa/63-kDa complex for vWF may be due to a simple addition of the affinities of the individual fragments. Alternatively, at least one of the fragments may have the appropriate conformation required for maximal vWF binding affinity only when the other fragment is present. To distinguish between these two possibilities, we tested the conformations of the acidic region and the C2 domain in the individual fragments as well as in the 14-kDa/63-kDa complex to determine if they are the identical.

The affinity of the 14-kDa fragment for anti-acidic region mAb NMC-VIII/10 (epitope, residues 1674–1684; Ref. 15) was compared with those for LCh and for the 14-kDa/63-kDa complex. The dissociation and association rate constants were derived for each ligand as before (data not shown). The similar <i>k</i><sub>on</sub>, <i>k</i><sub>off</sub>, and <i>K</i><sub>v</sub> values for the interaction of the 14-kDa fragment, 14-kDa/63-kDa complex and LCh with mAb NMC-VIII/10 (Table II) suggest that their acidic region conformations are identical regardless of the presence of the 1795–2332 residues. To test if the presence of the acidic region is required for the optimal vWF binding conformation of the C2 domain, the affinity of the LCh and the 14-kDa/63-kDa complex for immobilized anti-C2 mAb NMC-VIII/5 (epitope, residues 2170–2327), which is known to inhibit fVIII-vWF binding (22), was compared with that of A3-C1-C2, 63-kDa fragment, and C2. The lower affinity of NMC-VIII/5 for LCh derivatives lacking the acidic region than that for intact LCh or 14/63-kDa fragment suggests that removal of the acidic region produces a conformational change within the C2 domain regardless of whether C2 is attached to other regions of the LCh.

To exclude the possibility that the acidic region of the LCh is a part of the NMC-VIII/5 epitope and the reduced affinity of A3-C1-C2, C2, or 63-kDa fragment for NMC-VIII/5 is simply due to lack of this region, we tested the effect of NMC-VIII/5 on the C2 domain of intact LCh. The lower affinity of NMC-VIII/5 for LCh derivatives lacking the acidic region than that for intact LCh or 14/63-kDa fragment suggests that removal of the acidic region produces a conformational change within the C2 domain regardless of whether C2 is attached to other regions of the LCh.
The epitopes of monoclonal antibodies NMC-VIII/5 and NMC-VIII/10 correspond to VIII residues 2170–2327 and 1675–1684, respectively. The \( k_{on} \) and \( k_{off} \) values were derived from individual association and dissociation kinetic curves as described under “Experimental Procedures.” The \( K_d \) values were calculated as \( k_{off}/k_{on} \).

| Immobilized antibody | Soluble ligand | \( k_{on} \) \( M^{-1}s^{-1} \) | \( k_{off} \) \( s^{-1} \) | \( K_d \) \( nM \) |
|----------------------|---------------|-----------------|-----------------|-----------------|
| NMC-VIII/5           | LCh           | \( 1.03 \pm 0.12 \times 10^5 \) | \( 3.5 \pm 0.17 \times 10^{-5} \) | 0.344 \( \pm \) 0.045 |
| NMC-VIII/5           | A3-C1-C2      | \( 0.83 \pm 0.11 \times 10^5 \) | \( 4 \pm 0.3 \times 10^{-5} \) | 4.8 \( \pm \) 0.73 |
| NMC-VIII/5           | 14-kDa/63-kDa | \( 1.3 \pm 0.236 \times 10^5 \) | \( 3.3 \pm 0.14 \times 10^{-5} \) | 0.26 \( \pm \) 0.05 |
| NMC-VIII/5           | 63-kDa        | \( 1.0 \pm 0.087 \times 10^5 \) | \( 3.9 \pm 0.41 \times 10^{-4} \) | 3.3 \( \pm \) 0.41 |
| NMC-VIII/5           | C2            | \( 1.2 \pm 0.072 \times 10^5 \) | \( 3.2 \pm 0.22 \times 10^{-4} \) | 3.30 \( \pm \) 0.37 |
| NMC-VIII/10          | LCh           | \( 2.4 \pm 0.27 \times 10^3 \)  | \( 1.4 \pm 0.11 \times 10^{-3} \) | 5.9 \( \pm \) 0.82 |
| NMC-VIII/10          | 14-kDa/63-kDa | \( 2 \pm 0.16 \times 10^5 \) | \( 1.3 \pm 0.08 \times 10^{-3} \) | 5.7 \( \pm \) 0.63 |
| NMC-VIII/10          | 14-kDa        | \( 2.2 \pm 0.18 \times 10^3 \) | \( 1.4 \pm 0.06 \times 10^{-3} \) | 6.5 \( \pm \) 1.25 |

**DISCUSSION**

Several previous studies had identified mAbs that recognize epitopes in the acidic region of the VIII LCh (residues 1649–1689) and that also prevent the formation of the VIII-vWF complex (14–17). Deletion of the entire acidic region (1649–1689) eliminated vWF binding, whereas deletion up to 1668 did not (18). However, synthetic peptide 1673–1689 at a molar excess of 2,500 over VIII was not able to inhibit its binding to vWF (18). Our approach to this apparent contradiction was to assume that the synthetic peptide did not have the appropriate conformation for vWF binding and that a larger proteolytic LCh fragment might be more suitable. We were able to cleave the VIII LCh with S. aureus V8 protease to generate a 14-kDa fragment consisting of amino acids 1672 to approximately 1794, and we have shown in the present study that it does indeed bind to vWF.

In our study we used a surface plasmon resonance phenomenon for direct real time measurement of association and dissociation of unlabeled proteins and subsequent determination of the corresponding rate constants (42). Since one binding partner must be immobilized to a matrix, the kinetic parameters derived from optical biosensor kinetic measurements may potentially show deviations from those determined in a fluid phase assay. We were able to demonstrate, however, that the \( K_d \) values determined for VIII, LCh, 14-kDa, and C2 binding to vWF using the biosensor method (Table I) were similar to the \( K_d \) values of 0.38, 4.1, 84, and 470 nM for the respective fragments derived from the fluid phase assay in which binding between 125I-VIII and vWF was competed by unlabeled ligands. This result indicates that vWF was not altered by immobilization and therefore the kinetic parameters for binding of VIII and its derivatives to vWF derived in our experiments are valid. The high sensitivity of this technique and its capability to register fast dissociation kinetics has allowed us to determine up to 2,000-fold lower affinities of VIII proteolytic fragments for vWF than that of VIII.

An LCh derivatives lacking the acidic region (A3-C1-C2, C2, and 63-kDa fragment) had greatly reduced affinities for vWF (\( K_d \) 564–660 nM) compared with LCh (\( K_d \) 3.8 nM) (Table I). In addition, the similar affinities of A3-C1-C2, 63-kDa fragment, C2, and HCh/A3-C1-C2 for vWF indicated that the LCh contains no vWF binding site other than the one within C2. The 14-kDa fragment lacking the C2 domain also had a lower affinity (\( K_d \) 72 nM) for vWF than LCh. In contrast, the proteolytically cleaved but unassembled complex containing both the acidic region and C2 (14 kDa/63 kDa) had an affinity identical to that of the intact LCh. Reassociation of the 14-kDa/63-kDa complex from the individual 14- and 63-kDa fragments and the demonstration that its binding to vWF is similar to that of the original 14-kDa/63-kDa complex indicated that the above fragments were not altered by purification under denaturing conditions. The previous hypotheses that the LCh acidic region contains a vWF binding site and that both this region and the C2 domain are essential for high affinity binding are therefore confirmed. Our data also demonstrate that the two binding sites must be simultaneously present, although not necessarily covalently linked, for maximal affinity vWF binding to occur. Our data also demonstrate that the residues 1649–1671 are not involved in vWF binding, which is consistent with previous findings (18). We demonstrated that neither the acidic region peptide 1672–1689 nor the COOH-terminal 11.5-kDa fragment derived by the thrombin cleavage of 14-kDa fragment were able to bind vWF in biosensor experiments or inhibit VIII-vWF interaction in fluid phase assays. We hypothesize, therefore, that the acidic region vWF binding site extends COOH-terminal to the thrombin cleavage site at residue 1689 and that it is destroyed by thrombin cleavage. This hypothesis could explain why synthetic peptide 1673–1689 (18) did not bind vWF. The decreased affinity of the 14-kDa fragment affinity for vWF is due to a lower association rate constant (\( k_{on} \)) than that for LCh (Table I) since the dissociation rate constants determined for the 14-kDa fragment and LCh complexes with vWF were simi-
lar. For the C2 domain the $K_{d}$ value was 10-fold greater than that for LCh or 14-kDa fragment. These results suggest that the interaction of the LCh acidic region with vWF is the rate-determining step for the dissociation of LCh-vWF and fVIII-vWF complexes in the absence of thrombin activation. The loss of the acidic region leads to a 160-fold increased $K_{d}$ and a >1000-fold increased $K_{d}$ for HCh/A3-C1-C2 heterodimer binding to vWF compared with that of VIII. This would predict a similar reduction of fVIII affinity upon thrombin activation, allowing efficient fVIII binding to the phospholipid surface required for its maximal activity in the factor Xase enzyme complex.

We demonstrated that the LCh acidic region not only directly participates in vWF binding, but it is probably also required to maintain the normal conformation of the C2 binding site. We used anti-C2 mAb NMC-VIII/5, which prevents fVIII-vWF binding (19, 22), as a probe to detect possible conformational changes in C2 upon removal of the acidic region. The reduced affinities for NMC-VIII/5 of LCh derivatives lacking the acidic region indicated that such a change occurs. Similar affinities for NMC-VIII/5 for the LCh and the 14-kDa/63-kDa complex demonstrated that noncovalent association of the amino-terminal 14-kDa fragment with the carboxyl-terminal 63-kDa fragment appeared to be sufficient to maintain the C2 conformation similar to that within intact LCh. In contrast, the conformation of the acidic region does not depend on the presence of C2, since the affinity of the anti-acidic region mAb NMC-VIII/10 for the 14-kDa fragment and the LCh was similar.

Our results are consistent with the hypothesis that the light chain acidic region and C2 are in close proximity and together form one high affinity binding site for vWF. The computer modeling of the three-dimensional structure of the fVIII A domains, based on their structure in ceruloplasmin, predicts that the carboxyl and amino termini of A3 are in close proximity (43). In addition, the disulfide bond determined between Cys2021 and Cys2169 and that proposed between Cys2174 and Cys2326 (44) demonstrate that the amino and carboxyl termini of the C1 (residues 2019–2172) and C2 (residues 2173–2332) domains, respectively, are also spatially close. These findings suggest that the acidic region located at the amino terminus of the LCh and the carboxyl terminus of the C2 domain may also be close together in the three-dimensional structure of the LCh. Our findings that the LCh acidic region and C2 together form the high affinity vWF binding site is consistent with this model. The inhibition of high affinity fVIII binding to mature vWF residues 1–116 (45) by both anti-LCh acidic region and anti-C2 mAbs (21) would also fit this model.

The $K_{d}$ for LCh binding to vWF (3.8 nM) is 9.5 times higher than that for fVIII (0.4 nM), demonstrating that the HCh is required for the maximal affinity of fVIII for vWF. The higher affinity of fVIII-vWF interaction than that of LCh-vWF is mainly due to the lower dissociation rate of the former complex (Table I). The participation of the HCh in fVIII-vWF binding is indirect because the HCh itself did not bind to vWF in our experiments or as previously demonstrated by ultracentrifugation (8). Recently Sudhakar and Fay observed that dissociation of the HCh-LCh complex led to conformational changes in each chain (46). It is therefore possible that the LCh has to be associated with HCh to have the optimal conformation for its binding to vWF. Once the LCh acidic region is removed by thrombin cleavage, the HCh cannot enhance the binding of A3-C1-C2 to vWF.

Acknowledgment—We are grateful to Dr. Kenneth Ingham for critical review of the manuscript and helpful discussions.