Metal Ion-independent Association of Factor VIII Subunits and the Roles of Calcium and Copper Ions for Cofactor Activity and Inter-Subunit Affinity†

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ABSTRACT: Factor VIII circulates as a divalent metal ion-dependent heterodimer comprised of a light chain (LC) and a heavy chain (HC). Reassociation of factor VIII subunits was assessed using fluorescence energy transfer where LC and HC were labeled with acrylodan (Ac; fluorescence donor) and fluorescein-5-maleimide (Fl; fluorescence acceptor), respectively. The reduction of donor fluorescence due to the acceptor was used as an indicator of binding. Subunits associated with high affinity (K_d = 53.8 nM) in the absence of metal ion and presence of EDTA. However, this product showed no cofactor activity, as measured by a factor Xa generation assay. In the presence of 25 mM Ca^{2+}, no increase in the intersubunit affinity was observed (K_d = 48.7 nM) but specific activity of the cofactor was ∼30% that of native factor VIII. At saturating levels of Fl-HC relative to Ac-LC, donor fluorescence decreased to 79.3 and 73.5% of its original value in the absence and presence of Ca^{2+}, respectively. Thrombin cleaved the heterodimers that were associated in the absence or presence of Ca^{2+} with similar efficiency, indicating that the lack of activity was not the result of a defect in activation. Cu^{2+} (0.5 μM) increased the intersubunit affinity by ∼100 fold (K_d = 0.52 nM) and the specific activity to ∼60% of native factor VIII. The former effect was independent of Ca^{2+}, whereas the latter effect required Ca^{2+}. These results indicate that the intersubunit association in factor VIII is primarily metal-ion independent while divalent metal ions serve specific roles. Ca^{2+} appears essential to promote the active conformation of factor VIII while Cu^{2+} primarily enhances the intersubunit affinity.

Factor VIII, a plasma protein that participates in the blood coagulation cascade, is decreased or defective in individuals with hemophilia A. Factor VIII functions as a cofactor for the serine protease factor IXa in the surface-dependent conversion of zymogen factor X to the serine protease, factor Xa (*1*, *2*). Deficiency of factor VIII causes marked reduction of factor IXa activity and subsequent factor Xa generation.

Factor VIII is synthesized as an ∼300 kDa single chain precursor protein (*3*, *4*) with domain structure A1-A2-B-A3-C1-C2 (*5*). Factor VIII is processed to a series of divalent metal ion-linked heterodimers (*6*−*8*) by cleavage at the B-A3 junction, generating an HC minimally represented by the Al-A2 domains, that may possess all or part of the B domain; and a LC[1] consisting of the A3-C1-C2 domains. The A domains of factor VIII share homology with the A domains of factor V and the copper-binding protein, ceruloplasmin (*9*). Both factor V (*10*) and factor VIII (*11*) have been shown to contain 1 mol of copper. Recently, Cu^{+} has been identified as the ion form in factor VIII (*12*). The A1 and A3 domains participate in the divalent metal ion-dependent linkage. Both domains possess a single consensus type 1 copper binding site (*5*, *13*, *14*) and a consensus type 2 site has been proposed at the A1/A3 interface (*13*, *14*).

Factor VIII is inactivated by EDTA (*6*, *8*) and the component HC and LC can be isolated by ion-exchange chromatography (*15*, *16*). Factor VIII can be reconstituted by combining the isolated subunits in the presence of Ca^{2+} or Mn^{2+} (*16*−*18*). While high (mM) $^{2+}$ fail to support factor VIII reconstitution (*17*), low (μM) levels of Cu^{+} or $^{2+}$ stimulate reconstitution in the presence of Ca^{2+} (*12*, *19*) or Mn^{2+} (*19*).

Thrombin converts factor VIII to the active cofactor (factor VIIIa) by limited proteolysis (*20*). Thrombin cleaves factor VIII HC at Arg^{740}, which liberates the B domain (or fragments), and at Arg^{372}, which bisects the contiguous A1-A2 domains into the A1 and the A2 subunits. Cleavage of the LC at Arg^{1689} liberates an acidic rich region and creates a new NH_2-terminus. Thus, factor VIIIa is a heterotrimer of subunits designated as A1, A2, and A3-Cl-C2 (*21*, *22*). The A1 and A3-Cl-C2 subunits retain the divalent metal ion-dependent linkage and can be isolated as a stable Al/A3-Cl-C2 dimer. The A2 subunit is weakly associated with the dimer in a metal ion-independent interaction that is primarily electrostatic (*22*, *23*).

The mechanism of factor VIII HC and LC reassociation to form functional heterodimer and the role(s) of divalent

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[1] Abbreviations: HC, factor VIIII heavy chain; LC, factor VIII light chain; Ac-LC, LC modified with acrylodan; Fl-HC, HC modified with fluorescein-5-maleimide; bis-ANS, bisanilinonaphthalsulfonic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; BSA, bovine serum albumin; PS, phosphotidylserine, PC, phosphotidylcholine; PE, phosphotidylethanolamine; and SDS−PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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metal ion(s) in this interaction remain largely unknown. In this report, we demonstrate differential effects of metal ions on factor VIII subunit association and generation of cofactor activity. Analyses employed a combination of fluorescence energy transfer and activity assay in order to correlate structural changes with function. In the absence of metal ion, LC and HC combine to form an inactive dimer. Ca^{2+} has little effect on intersubunit affinity, yet converts the inactive dimer to an active, although somewhat lower specific activity form. Alternatively, Cu^{2+} enhances the intersubunit affinity ~100-fold but yields a dimer that lacks activity. However, the presence of both metal ions results in a high intersubunit affinity and yields a high specific activity factor VIII.

MATERIALS AND METHODS

Reagents. Recombinant factor VIII preparations (Kogenate) were a gift from Dr. Jim Brown of Bayer Corporation (Berkeley, CA). Purified recombinant factor VIII was also a generous gift from Debbie Pittman of the Genetics Institute (Cambridge, MA). Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using *N*-octylglucoside as described previously (*24*). The reagents α-thrombin, factor IXa*β*, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin and phospholipids (Sigma, St. Louis, MO), and the chromogenic Xa substrate S-2765 (*N*-α-benzyloxycarbonyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide-dihydrochloride; DiaPharm Group, Westchester, OH) were purchased from the indicated vendors. Acrylodan, and fluorescein-5-maleimide were purchased from Molecular Probes (Eugene, OR).

Isolation of FVIII Subunits. Factor VIII LC and HC were isolated following treatment of factor VIII with EDTA and subsequent chromatography using Mono S and Mono Q columns as described (*16*) with minor modification. Purified factor VIII subunits were dialyzed into 10 mM MES, 0.3 M NaCl, 0.01% Tween-20, 0.1 mM EDTA, pH 6.5 (buffer A) containing Chelex 100 and stored at −80 °C.

Factor Xa Generation Assays. The rate of conversion of factor X to factor Xa was monitored in a purified system (*25*). Factor VIII subunit reconstitution was performed by mixing LC and HC at the indicated concentrations in the presence or absence of metal ions in 10 mM MES, 0.3 M NaCl, 0.01% Tween-20, 0.1 mg/mL BSA, pH 6.5 (buffer B) at 25 °C for 18 h. The concentration of reconstituted factor VIII was based upon the concentration of the limiting subunit (LC). Native factor VIII or reconstituted factor VIII (1−5 nM) was activated with 20 nM α-thrombin in 20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween-20, pH 7.2 in the presence of 0.1 mg/mL BSA and 10 μM PSPCPE vesicles for 1 min. This reaction was stopped by addition of 20 units/mL hirudin and the resultant factor VIIIa was reacted with factor IXa (20−40 nM) for 30 s. Time course reactions were initiated with the addition of 500 nM factor X. Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (50 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined by addition of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were read at 405 nm using a Vmax microtiter plate reader (Molecular Devices, Sunnyvale, CA). All reactions were run at 25 °C. The activity was expressed as the amount of factor Xa generated (nM) per minute and converted to the value per nM LC. Activity as a function of applied HC concentration was plotted and the data from all the samples were fitted to the quadratic equation by nonlinear least-squares regression,

$$A = k(L_0 + H_0 + K_d - \sqrt{(L_0 + H_0 + K_d)^2 - 4L_0H_0})$$

where A is activity (nM/min/nM LC), L_0 is the LC concentration, H_0 is the HC concentration, K_d is the dissociation constant, and k is a constant.

Fluorescent Labeling of Factor VIII Subunits. Acrylodan (excitation/emission wavelength = 391/500 nm) labeled factor VIII light chain (Ac-LC) and fluorescein (excitation/emission wavelength = 492/515 nm) labeled factor VIII heavy chain (Fl-HC) were prepared by sulfhydryl specific protein modification. LC (512 μg/mL) in 20 mM HEPES, 0.25 M NaCl, 1 mM $CaCl_2$, 0.01% Tween-20, pH 7.2, was reacted with a 6-fold molar excess acrylodan overnight at 4 °C in the dark. This level of acrylodan was used to minimize side reactions of the fluorophore with amino groups (*26*). Unbound fluorophore was removed by exhaustive dialysis (>24 h) at 4 °C against buffer A. Direct labeling of HC with fluorescein-5-maleimide failed to yield active subunit as judged by reconstitution assay. For that reason, intact factor VIII (1 mg/mL) was labeled with a 50-fold molar excess fluorescein-5-maleimide overnight at 4 °C in the dark. Fl-HC was purified following EDTA-treatment of Fl-factor VIII as described above.

Fluorescence Energy Transfer. One hundred nanomolar LC (or Ac-LC) and 0−300 nM HC (or Fl-HC) were reconstituted at 25 °C overnight (>18 h) in buffer B in the presence of either 25 mM $CaCl_2$, 0.5 μM Cu^{2+}, 25 mM Ca^{2+} plus 0.5 μM Cu^{2+}, or 5 mM EDTA. The optimum pH of 6.5 for the subunit association was determined from factor Xa generation assays with the Ca^{2+}-containing heterodimers. In the case of the reconstitution with 25 mM Ca^{2+} plus 0.5 μM Cu^{2+}, factor VIII subunits were first reacted with 0.5 μM Cu^{2+} for 2 h followed by 25 mM $CaCl_2$ for an additional 16 h. Fluorescence measurements were performed using an Aminco-Bowman Series 2 Luminescence Spectrometer (Thermo Spectronic, Rochester, NY) at room temperature at the excitation wavelength of 395 and 4 nm bandwidth. Emission fluorescence was monitored at 420−550 nm and all spectra were corrected for background. Energy transfer between donor and acceptor fluorophores is fast relative to factor VIII subunit association. The relative fluorescence (F) was calculated as follows:

$$F(\%) = \frac{[F_{DA} - (F_{DA} - F_A)]}{F_D} \times 100$$

where F_D is the fluorescence intensity of donor plus unlabeled acceptor (Ac-LC/HC), F_{DA} is the fluorescence intensity of labeled donor plus labeled acceptor (Ac-LC/Fl-HC), and F_A is the fluorescence intensity of unlabeled donor plus labeled acceptor (LC/Fl-HC). Integrated fluorescence intensities at 450−490 nm from triplicate readings were averaged for each sample and a single value for relative fluorescence was calculated by averaging values from six separate samples. Control experiments showed no effects of

the divalent metal ions at the concentrations employed on the fluorescence properties of the isolated subunits.

Relative fluorescence as a function of HC concentration was plotted and the data from all the samples were fitted to the quadratic equation by nonlinear least-squares regression (*27*, *28*) using the formula,

$$F = 100 - k(L_0 + H_0 + K_d - \sqrt{(L_0 + H_0 + K_d)^2 - 4L_0H_0})$$

where F is the relative fluorescence (%), L_0 is the concentration, H_0 is the HC concentration, K_d is the dissociation constant, and k is a constant.

Electrophoresis. SDS−PAGE was performed using the buffer system of Laemmli (*29*). Gels were cast on a mini gel apparatus (Bio-Rad) and electrophoresis was for 50 min at 200 V. Gels were scanned using the Storm gel-and-blot Imaging System (Storm 860, Molecular Dynamics, Sunnyvale, CA) after staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL). The fluorescein-labeled molecular weight marker (Sigma) used consisted of myosin (M_r = 205 000), β-galactosidase (116 000), bovine serum albumin (66 000), alcohol dehydrogenase (39 000), carbonic anhydrase (29 000), and trypsin inhibitor (20 100).

Statistical Analysis. Nonlinear least-squares regression was performed using Kaleidagraph (Synergy, Reading PA) and each parameter and its standard error were obtained. Since degrees of freedom are the same between samples, the values of t were estimated by the following formula:

$$t = \frac{(P_1 - P_2)}{\sqrt{S_1^2 + S_2^2}}$$

where P_1 and P_2 are best-fit value of each parameters, S_1 and S_2 are their standard errors. The percentage points (probability) for the Student's t-distribution were calculated using Microsoft Excel spread sheet.

RESULTS

Fluorophore Labeling of Factor VIII Subunits. Combining factor VIII LC and HC in the absence of added divalent metal ion fails to reconstitute factor VIII activity (*17*). However, it is unclear whether the subunits assemble under these conditions to yield an inactive heterodimer. To assess subunit association and the role of selected metal ions, we employed an assay using fluorescence resonance energy transfer. Fluorophore selection was based upon spectral properties and labeling chemistry, the latter made use of the presence of a free cysteine residue identified in each A domain of factor VIII (*30*). Factor VIII LC labeled with the fluorescence donor, acrylodan, retained ~90% activity based upon reconstitution analysis using unlabeled HC under control conditions. The probe was incorporated at an estimated molar ratio of 0.89 (acrylodan:LC) based on the fluorescence comparison with an acrylodan-labeled A2 subunit (*31*) (data not shown). Labeling the isolated factor VIII HC with the fluorescence acceptor, fluorescein-5-maleimide, was problematic yielding inactive subunit. This situation was similar to an earlier experience attempting to label the isolated A2 subunit of factor VIIIa (*31*). However, active Fl-HC was obtained by first labeling the intact factor VIII, followed by isolation of the separated subunit. Modification of factor VIII with the fluorophore showed little if any effect on cofactor activity (data not shown). The Fl-HC prepared in this manner retained >80% activity based upon reconstitution assays. The reason for loss of activity following the labeling reaction of the isolated HC is not known, but may reflect side reactions of the fluorescein with residues, possibly within the A2 domain, that are not exposed in the factor VIII heterodimer. Based on the molar extinction coefficient for fluorescein (83 000 at 495 nm), 1.88 mol of fluorescein was incorporated per mol HC. This result suggested that fluorophore reacted with the free thiol groups localized to the A1 (Cys310) and the A2 (Cys692) domains (*30*). To evaluate the labeling relative to factor VIII domains, the Fl-HC was reacted with thrombin, subjected to SDS−PAGE and resultant bands visualized under fluorescent light (Figure 1). The fluorescence intensity from the bands corresponding to A1 and A2 subunits (Figure 1B) was quantitated and standardized using the densitometric value from each band in the gel in Figure 1A. From that result we obtained a fluorescence incorporation ratio of 1.16 for Fl-A1 and 0.72 for Fl-A2 (1.6 for A1:A2). The concentration of fluorescein-5-maleimide did not affect the efficiency of A1-labeling but did effect that of A2 labeling, such that incorporation into the A2 domain increased when the fluorescein-5-maleimide concentration was increased relative to HC concentration (data not shown). These observations suggested that labeling was probably localized to a single site on each domain and the site within A1 was more accessible and/or reactive than that within A2.

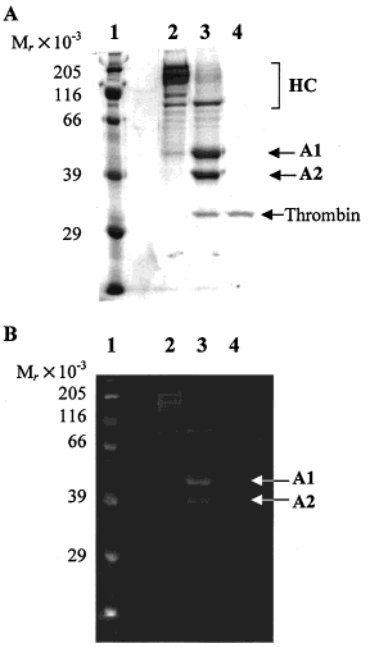

FIGURE 1: SDS−polyacrylamide gel electrophoresis of fluorescein-labeled factor VIII heavy chain. Fluorescein-labeled factor VIII heavy chain was subjected to electrophoresis using a 10% polyacrylamide gel and visualized by GelCode stain (A) and fluorescence imaging (B). Fl-HC was cleaved with thrombin (400 nM) for 25 min at room temperature. Samples are fluorescein-labeled molecular weight markers (lane 1), Fl-HC (lane 2), thrombin cleaved Fl-HC (lane 3) and thrombin alone (lane 4).

Association of Factor VIII Subunits in the Presence or Absence of Metal Ions as Determined by Fluorescence Energy Transfer. Fluorescence emission spectra of repre-

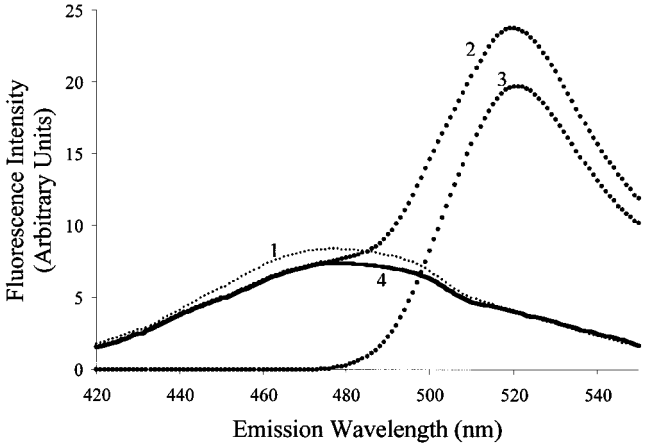

FIGURE 2: Fluorescence energy transfer using Ac-LC and Fl-HC. Subunit concentrations were 100 nM Ac-LC and 200 nM Fl-HC, and samples were excited at 395 nm (4 nm bandwidth). The reaction conditions are described in the Materials and Methods. Emission spectra, corrected for background fluorescence are (1) Ac-LC + unmodified HC; (2) Ac-LC + Fl-HC; (3) unmodified LC + Fl-HC; and (4) spectrum 2 − spectrum 3. Relative fluorescence was calculated from (area of spectrum 4)/(the area of spectrum 1), integrating the spectra between 450 and 490 nm. Fluorescence intensity is in arbitrary units.

sentative samples excited at 395 nm are shown in Figure 2. The reduction of the fluorescence of acrylodan due to energy transfer was observed in the sample where Ac-LC was associated with Fl-HC. Under these reactions conditions (100 nM Ac-LC and 200 nM Fl-HC in the absence of divalent metal ion) the extent of fluorescence quenching was ∼15%. Increasing the concentration of the fluorescein acceptor, Fl-HC, yielded a saturable increase in the extent of quenching (see below).

Figure 3 shows the reduction of the relative fluorescence of Ac-LC (100 nM) due to quenching by increasing concentrations of Fl-HC following reaction for 18 h. The reduction in relative fluorescence was proportional to the amount of binding Ac-LC and Fl-HC and this value was used to calculate equilibrium dissociation constants (*32*). For these reactions, salt concentration was optimized at 0.3 M NaCl, which is similar to the value employed in our early studies of factor VIII reconstitution (*16*, *17*). At 0.1 M NaCl, we observed precipitation at high concentrations of LC which subsequently could be dissolved by 0.3 M NaCl. However, some aggregation was likely present at lower concentrations of LC as judged by an unrealistically high level of quenching at low concentrations of Fl-HC (<50 nM) (data not shown), resulting in a calculated stoichiometry of LC:HC = 4−5:1 from the fitted curve. On the other hand, at 0.4 M NaCl, the extent of quenching at a given subunit concentration decreased suggesting inhibition of subunit reassociation.

Reactions run in the absence of exogenous divalent metal ion (Figure 3A) included EDTA (5 mM) to chelate any trace metal contamination in the reaction solutions. Under these conditions a K_d of 53.8 ± 14.2 nM was calculated from the donor quenching data (see Table 1). Interestingly, this value was similar to that obtained for reactions containing 25 mM $CaCl_2$ (48.7 ± 15.4 nM), indicating that Ca^{2+} did not influence the intersubunit affinity. The relative fluorescence at the saturation of LC was significantly different between the complex without metal ion (79.3 ± 1.4%) and that with Ca^{2+} (73.5 ± 2.0%, $p < 0.05$). This observation suggested

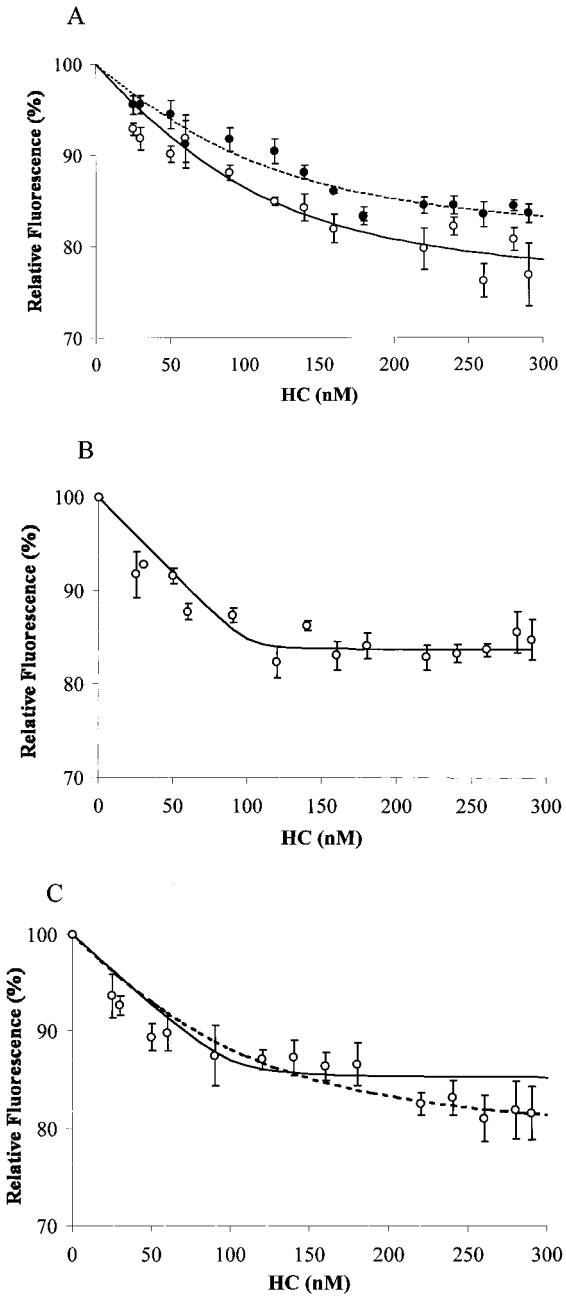

FIGURE 3: Donor fluorescence quenching as a result of subunit reassociation at equilibrium in the presence of 25 mM Ca^{2+} or 5 mM EDTA (A), 0.5 μM Cu^{2+}(B), or 0.5 μM Cu^{2+} plus 25 mM Ca^{2+} (C). The relative fluorescence represents a ratio of fluorescence intensities as described in the legend to Figure 2 (spectrum 4/spectrum 1) integrated between 450 and 490 nm. The relative fluorescence from 100 nM Ac-LC reacted with the indicated levels of Fl-HC were determined in the presence of 25 mM Ca^{2+} (A, open circles) or 5 mM EDTA (A, closed circles), 0.5 μM Cu^{2+} (B), or 0.5 μM Cu^{2+} plus 25 mM Ca^{2+} (C). Each point represents the average value of 6 separate samples. Lines were drawn by curve fitting using the equilibrium equation described in Materials and Methods.

a Ca^{2+}-dependent conformational change yielding a closer interfluorophore spatial separation in the latter condition. However, given the heterogeneity of HC labeling, it is not clear whether the fluorophore-labeled residue in the LC became more proximal to the labeled residue in the A1 and/or the A2 domain.

Factor VIII has been shown to contain a single copper ion (*11*) [Cu^+ (*12*)]. However, given the tendency of Cu^+ to

Table 1: Estimated Equilibrium Parameters for Factor VIII Light Chain and Heavy Chain[a]

condition	K_d[b] (nM)	RF max[c] (%)	activity[d] (nM Xa/min/nM LC)
HC/LC			
+ 5 mM EDTA	53.8 ± 14.2	79.3 ± 1.4	<0.1
+ 25 mM Ca^{2+}	48.7 ± 15.4	73.5 ± 2.0[e]	15.7 ± 1.5
+ 0.5 μM Cu^{2+}	0.52 ± 1.25[f]	83.6 ± 0.5[g]	<0.3
+ 0.5 μM Cu^{2+} plus 25 mM Ca^{2+}			30.4 ± 1.9
(high affinity)	1.96 ± 4.36	85.2 ± 1.4	
(low affinity)	40.4 ± 58.0	77.9 ± 5.7	

[a] Reconstitution reactions were carried out in buffer containing 10 mM MES, pH 6.5, 0.3 M NaCl, 0.01% Tween-20, 0.1 mg/mL BSA in the presence or absence of metal ions as indicated for 18 h at 25 °C, and assessed by fluorescence energy transfer and factor Xa generation as described in the Materials and Methods. [b] Values were determined from data shown in Figure 3. [c] Relative fluorescence (RF) of Ac-LC at saturating Fl-HC determined by fluorescence energy transfer. [d] Activity for factor VIII reconstituted at saturating HC relative to LC determined a factor Xa generation assay. [e] $p < 0.05$ compared with HC/LC + 5 mM EDTA. [f] $p < 0.0005$ compared with HC/LC + 5 mM EDTA. [g] $p < 0.005$ compared with HC/LC + 5 mM EDTA.

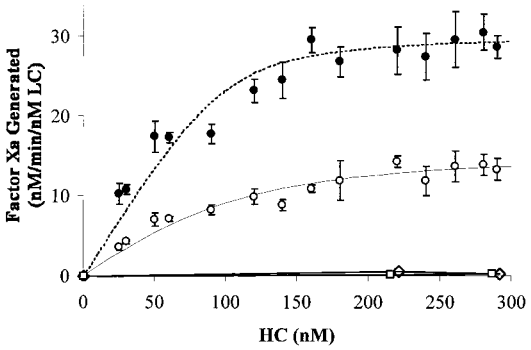

FIGURE 4: Factor VIII activity following subunit reassociation. Reconstituted factor VIII activity using 100 nM (Ac-)LC and the indicated amount of (Fl-)HC in the presence of 25 mM Ca^{2+} (open circles), 0.5 μM Cu^{2+} (squares), 0.5 μM Cu^{2+} plus 25 mM Ca^{2+} (closed circles), or no added metal ion (diamonds) was measured using the factor Xa generation assay as described in Materials and Methods. Each point represents the average value of four determinations. Curves were drawn from the fitted data using the equilibrium equation described in Materials and Methods.

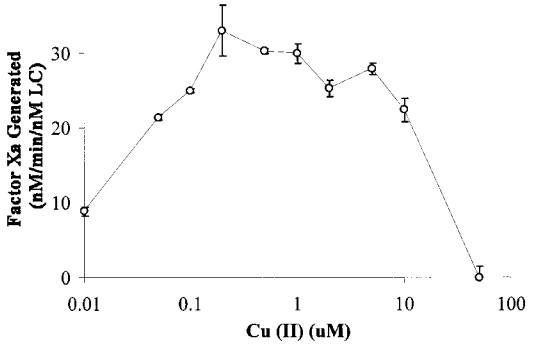

FIGURE 5: Effect of Cu^{2+} on reconstituted factor VIII activity. Factor VIII was reconstituted using 100 nM each LC and HC in the presence of 25 mM Ca^{2+} plus the indicated levels of Cu^{2+}. Resultant cofactor activity was determined by factor Xa generation assays as described in the Materials and Methods.

oxidize, our studies employed Cu^{2+}, which also has been shown to stimulate factor VIII reassociation (*12*, *19*). The presence of a low concentration of Cu^{2+} (0.5 μM) resulted in an ~100 fold increase in the intersubunit affinity (K_d = 0.52 ± 1.25 nM, Figure 3B, Table 1). This result indicated that Cu^{2+} had a marked effect on chain association that was not observed for Ca^{2+}, even though the latter is required for generation of functional activity (*19*). The level of donor quenching observed at saturating Fl-HC in the presence of Cu^{2+} (83.6 ± 0.5%; $p < 0.005$) was somewhat less than observed in the other conditions suggesting an average increase in the interfluorophore distance. The concentration of Cu^{2+} employed for these studies was based upon activity data as well as the effects of Cu^{2+} on fluorescence emission. While cofactor specific activity was enhanced over a broad concentration range of Cu^{2+} (0.02−5 μM, see Figure 5 below), fluorescence emission intensity of Ac-LC and Fl-HC were reduced by ~50% at 10 and 5 μM Cu^{2+}, respectively (data not shown). However, no alterations in emission spectra were observed at Cu^{2+} concentrations <0.6 μM.

When HC and LC were reassociated in the presence of both 0.5 μM Cu^{2+} and 25 mM Ca^{2+} (Figure 3C), the equilibrium titration curve gave the appearance of a biphasic pattern. In this series of reactions, HC and LC were reacted with 0.5 μM Cu^{2+} for 2 h followed by the addition of 25 mM Ca^{2+} and the incubation continued for an additional 16 h. Data were subjected to nonlinear regression performed according to the formula described in Methods. The high affinity component observed following titration with Fl-HC was calculated from data derived from the 0−180 nM HC concentrations, which yielded an apparent K_d of 1.96 ± 4.36 nM with a maximal quenching level of 85.2 ± 1.4%. The low affinity component was calculated from data derived from 220 to 290 nM HC and indicated an apparent K_d of 40.4 ± 58.0 nM with a maximal quenching level of 77.9 ± 5.7%. Given the high standard deviations obtained, we cannot state the K_d values with high confidence. However, these values taken together with the levels of maximal quenching for the high and low affinity interactions were consistent with values obtained in the presence of Cu^{2+} alone and Ca^{2+} alone, respectively. These results may suggest that the subunits reassociate by selective metal ion-induced conformations, as indicated by the differential levels of maximal donor quenching, that possess different intersubunit affinities.

Factor VIII Activity Reconstitution in the Presence or Absence of Metal Ions. Cofactor activity of the reconstituted factor VIII molecules was assessed using a factor Xa generation assay. Reconstitutions were performed for 18 h at 25 °C with LC concentration held constant at 100 nM and varying levels of HC. Due to the dependence of Ca^{2+} for the assay, EDTA was omitted from the reactions run in the absence of metal ion. In the presence of Ca^{2+}, activity increased in response to increasing HC (Figure 4). Activity values obtained using either labeled or unlabeled subunits were equivalent and all data were averaged. Maximal activity observed for factor VIII reconstituted in the presence of Ca^{2+} was 15.7 ± 1.53 nM/min/nM LC (Table 1). This value was

~30% that of native factor VIII (53.5 ± 6.0 nM/min/nM LC, data not shown), suggesting a reduced specific activity for factor VIII reconstituted under these conditions. However, in the absence of added Ca^{2+}, no detectable cofactor activity was observed at HC concentrations that saturated LC.

Effects of Cu^{2+} on Factor VIII Reconstitution. Earlier results from our laboratory indicated that Cu^{2+} alone did not promote reconstitution of factor VIII (*17*) and in fact, high levels were inhibitory (*19*). An experiment was performed to assess a wide range of Cu^{2+} concentrations on cofactor reconstitution assays run in the presence of 25 mM Ca^{2+} and 100 nM factor VIII subunits (Figure 5). Assuming a K_d of ~50 nM, these concentrations of subunits predict ~50% heterodimer formed. Results from this study showed that low levels of Cu^{2+} (0.01 to ~5 μM) increased factor Xa generation rates up to ~3-fold, whereas higher concentrations (>10 μM) yielded significant inhibition and led to complete inhibition of regeneration of cofactor activity. These results suggest at least two types of Cu^{2+} sites in the subunits showing differential affinities. Binding at the high affinity site (estimated $K_d \approx 10-100$ nM) stimulates subunit reassociation and cofactor activity; whereas binding at the low affinity site (estimated $K_d \approx 10-100$ μM) results in loss of activity. Upon the basis of these observations, 0.5 μM Cu^{2+} was used for energy transfer and activity assays.

When reconstituted factor VIII activity was assessed as a function of HC concentration in the presence of both 25 mM Ca^{2+} and 0.5 μM Cu^{2+}, we observed an increase in specific activity of the reconstituted factor VIII (30.4 ± 1.86 nM/min/nM LC) to a value that was approximately 60% of that observed for native factor VIII (Figure 4). On the other hand, reactions run in the presence of Cu^{2+} but lacking Ca^{2+} showed no factor VIII activity even at saturating levels of HC, similar to earlier observations (*19*). Taken together with results presented above, Cu^{2+} appears to both increase the intersubunit affinity and stimulate specific activity of the reconstituted factor VIII. However, the activity component of this effect is dependent upon the presence of Ca^{2+}.

Thrombin Cleavage of Factor VIII Reassociated in the Absence or Presence of Ca^{2+}. Thrombin converts factor VIII to the active cofactor (factor VIIIa) by limited proteolysis (*33*) and this conversion is essential for generation of cofactor activity. Thus one possibility for the disparate activities of heterodimers formed in the absence versus presence of metal ion is altered rates of cofactor activation. We examined whether thrombin cleaved factor VIII reconstituted without metal ion at the same efficiency as that formed with Ca^{2+} (Figure 6). Factor VIII reconstituted in the presence of Ca^{2+} or in the presence of EDTA was treated with thrombin and products were examined at following SDS−PAGE. Results indicated that the extent of cleavage of the HC to yield A1 and A2 subunits and cleavage of the LC to yield the A3-C1-C2 subunit were indistinguishable for the two substrates. Furthermore, the extent of products generated from the reconstituted factor VIII was similar to that observed using native factor VIII (results not shown). Thus, altered thrombin interaction with factor VIII formed in the absence of Ca^{2+} did not account for its lack of activity.

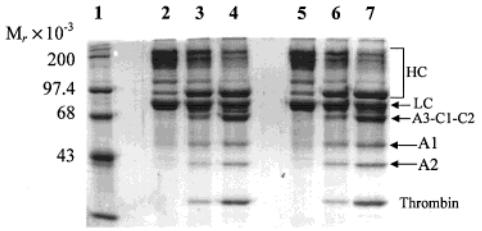

FIGURE 6: Thrombin cleavage of factor VIII reassociated in the presence of 25 mM Ca^{2+} or 5 mM EDTA. HC (75 μg/mL) and LC (40 μg/mL) were reconstituted in the presence of 25 mM Ca^{2+} (lanes 2−4) or 5 mM EDTA (lanes 5−7) and subsequently reacted in the absence (lanes 2 and 5) or presence of 40 nM (lanes 3 and 6) or 200 nM (lanes 4 and 7) thrombin for 2 min at room temperature. Electrophoresis was performed using a 10% polyacrylamide gel and visualized by GelCode stain. The molecular weight markers are shown in lane 1.

DISCUSSION

In this report, we demonstrate a metal ion-independent association of factor VIII HC and LC and dissect the contributions of Ca^{2+} and Cu^{2+} with regard to intersubunit affinity and the generation of cofactor activity. Subunit reassociation was monitored using a fluorescence energy transfer assay to assess physical association of the HC and LC, whereas a factor Xa generation assay was employed to assess function of the products. While HC and LC associated in the absence of divalent metal ion, the resulting heterodimers were inactive. Inclusion of Ca^{2+} did not increase the intersubunit affinity but yielded a factor VIII that possessed ~30% the activity of native material, whereas Cu^{2+} did not by itself contribute to activity but rather increased the Ca^{2+}-dependent specific activity ~2-fold and enhanced the intersubunit affinity by ~100-fold (K_d = 0.5 nM). Interestingly, a significant fraction of the binding energy in the association of factor VIII subunits is derived from the metal ion-independent interaction. Under these conditions, a K_d of 54 nM was determined, reflecting a thermodynamic stability of ~10 kcal/mol. This value is ~80% that obtained in the presence of Cu^{2+} (12.6 kcal/mol). Thus, in the presence of both metal ions, a high affinity and high specific activity product was obtained.

Early studies on the reconstitution of factor VIII from isolated HC and LC showed a strict requirement for Ca^{2+} or Mn^{2+} for the regeneration of cofactor activity (*17*, *18*). This requirement was believed necessary for the reassociation of chains, as has been demonstrated for factor Va. Indeed, the rigorous fluorescence studies of Krishnaswamy et al. (*32*) showed energy transfer between fluorophore-labeled factor Va HC and LC, as well as regeneration of activity, were dependent upon the presence of Ca^{2+} and resulted in an intersubunit K_d of ~6 nM. The reason for the lack of detectable cofactor activity in the metal ion-independent factor VIII dimer is not clear. The similarity of thrombin cleavage of factor VIII formed in the absence or presence of metal ion suggests that cofactor activation is not impaired. However, differences in the extents of donor quenching for the two substrates suggest that changes in heterodimer conformation in response to the presence of metal ion may be critical to proper interaction with factor IXa. Thus, the roles of Ca^{2+} in reassociation of the homologous proteins are not identical.

While factor VIII activity can be regenerated in the presence of Ca^{2+} or Mn^{2+} alone, maximal specific activity observed under these conditions is fractional (<1 unit/μg factor VIII) relative to the specific activity of native factor VIII (~3−5 units/μg). Early studies (*16*, *17*) attributed this

result to incomplete reassociation and/or the lability of subunit(s). More recently, we showed that Cu^{2+} enhanced the specific activity of factor VIII heterodimers reconstituted in the presence of Ca^{2+} or Mn^{2+} by ~3-fold (*19*). The effect of Cu^{2+} in the latter study suggested a synergy in the presence of the two divalent metal ions.

The physiologic role for copper in the observed affinity increase for the inter-factor VIII chain interaction is not yet clear. Given the factor VIII concentration in plasma is ~1 nM, the inter-factor VIII subunit affinity (K_d = 0.52 nM) may identify an important role for this ion in promoting the heterodimeric structure. The affinity of HC and LC is also regulated by von Willebrand factor (vWF) which increases the rate of reconstitution (*17*) presumably by acting as a surface on which factor VIII subunits were reconstituted. Recently, Saenko et al. (*34*) showed that the association rate constant for factor VIII subunits was increased ~5-fold in the presence of vWF when subunit reassociation was measured in the presence of either Ca^{2+} or Mn^{2+}. The vWF showed no effect in dissociation rates for the factor VIII forms, thereby indicating an overall ~5-fold increase in affinity between HC and LC in the presence of vWF (K_d = 15.5 nM) compared with its absence (K_d = 91.3 nM). While the K_d value obtained by these investigators in the absence of vWF was similar to the value we observed in the presence of Ca^{2+} (~54 nM), the role of copper ion was not explored in that study. We speculate that since Cu^{2+} and vWF likely enhance the intersubunit affinity by different mechanisms, their effects may be additive.

EDTA-treatment of factor VIII results in activity loss (*6*, *8*) and subsequent ion-exchange chromatography effectively separates factor VIII into LC and HC (*15*, *16*). Upon the basis of the current study, removal of bound metal ion-(s) (Cu^{+}) would result in an ~100-fold reduction in the intersubunit affinity. Subsequent adsorption of this material to Mono S, a cation exchanger, binds LC tightly whereas HC does not strongly interact with this resin and is washed away at moderate salt. We have observed that subunit separation following EDTA-treatment is more efficient at lower factor VIII concentrations (<1 μM), whereas application of the treated factor VIII to the column at relatively higher protein concentrations yields LC with increased HC contamination. This observation likely is a direct reflection the metal ion-independent affinity of factor VIII subunits.

Localization of metal ion binding sites within the factor heterodimer remains to be determined. While the number of bound Ca^{2+} is unknown, its presence appears essential to cofactor function. We observed that Ca^{2+} converted the inactive factor VIII heterodimer to an active one. This conversion was possibly the result of changes in the dimer conformation as judged by a decrease in the interfluorophore separation of LC and HC from the donor quenching data. Ca^{2+} typically binds at carboxylate moieties (*35*). Earlier results from our laboratory indicated that Ca^{2+}, and to a greater extent LC plus Ca^{2+}, could displace bound bis-ANS from an exposed hydrophobic pocket in the factor VIII HC (*36*). Taken together, these observations suggest the proximity of Ca^{2+} sites to hydrophobic region(s) that participate in the intersubunit interaction. Thus Ca^{2+} bound to this site(s) could alter the HC/LC orientation in such as manner as to somewhat alter the spatial separation of the fluorophores and generate cofactor function. This conclusion is consistent with results of Laue et al. (*37*) who examined intrinsic fluorescence during Ca^{2+}-mediated reconstitution of factor Va from isolated chains. Their results suggested that association of Ca^{2+} to form factor Va did not result in significant restructuring of the subunits, but rather Ca^{2+} did elicit modest changes in tertiary and quaternary structure that correlated with regeneration of activity.

EPR experiments have identified a single bound copper ion (*11*), and this ion is not associated with the isolated HC and LC following subunit dissociation. Subsequent analysis showed the ion to be Cu^{+} (*12*). The location of the bound Cu^{+} in factor VIII is controversial. Consensus type I copper sites have been identified in the A1 domain (His265, Cys310, His315, and Met320) and the A3 domain (His1954, Cys2000, His2005, and Met2010) (*5*). Modeling studies (*14*) suggest that a single consensus type II copper site is formed at the A1/A3 domainal junction involving His99 in A1 and His1957 in A3 plus the oxygen atom of a water molecule H-bonded to Tyr106 and Ala100. Based upon mutagenesis studies, Tagliavacca et al. (*12*) suggested that the type I site in A1 contained the bound Cu^{+}. The basis for this conclusion was marked reduction in the specific activity of a transiently expressed Cys310 to Ser factor VIII. These investigators also showed that conversion of Cys2000 to Ser and His1957 to Ala did not affect factor VIII specific activity, whereas His99 to Ala yielded ~25% the control value.

In the present study, thiol-specific fluorophores were employed to modify factor VIII subunits. Fluorescein-5-maleimide-modified factor VIII retained activity and the HC isolated from this factor VIII (Fl-HC) showed >80% native activity in a reconstitution assay. Thrombin cleavage showed both A1 and A2 domains were labeled with the fluorophore. Similarly, acrylodan-modified LC retained >90% native activity. We also observed retention of subunit activity in an earlier study employing different fluorophores possessing similar sulfhydryl specificity (*16*). Assuming the fluorophore-modified residues are the free thiol groups identified in A1 (Cys310) and A3 (Cys2000) domains (*30*), the incorporation of fluorophore would likely preclude Cu^{2+} binding at the two consensus type I site since these Cys residues contribute to coordination of the ion. Given our observations that Cu^{2+} primarily enhances the intersubunit affinity, we speculate that this interaction is mediated in a Cys-independent manner by the consensus type II copper site at the A1/A3 domainal junction.

In summary, the results of these studies support a model where the association of factor VIII subunit is largely governed by metal ion-independent interactions. We suggest the Ca^{2+} ion(s), localized to acidic regions(s) at or near surface exposed hydrophobic residues, participate in the intersubunit association and these ions are essential for conformational changes that yield the active cofactor. We also speculate that the bound Cu^{+} is coordinated at the type II site at the A1/A3 domainal junction. Thus, the presence of Cu^{+} likely bridges the A1 and A3 domains, thereby enhancing intersubunit affinity by ~100 fold, an effect that may be critical in maintaining the heterodimeric structure at physiologic factor VIII concentrations.

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