Ca^{2+} Binding to Both the Heavy and Light Chains of Factor VIII Is Required for Cofactor Activity†

Hironao Wakabayashi, Kyla M. Schmidt, and Philip J. Fay*

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, New York 14642

Received January 24, 2002; Revised Manuscript Received April 3, 2002

ABSTRACT: Previously, we demonstrated that Ca^{2+} was necessary for the generation of cofactor activity following reconstitution of factor VIII from its isolated light chain (LC) and heavy chain (HC) but that Ca^{2+} did not affect HC-LC binding affinity (Wakabayashi et al. (2001) *Biochemistry* 40, 10293−10300). Titration of EDTA-treated factor VIII with Ca^{2+} followed by factor Xa generation assay showed a two-site binding pattern, with indicated high-affinity ($K_d = 8.9 \pm 1.8$ μM) and low-affinity ($K_d = 4.0 \pm 0.6$ mM) sites. Analysis by equilibrium dialysis using ^{45}Ca and <400 μM free Ca^{2+} verified a high-affinity binding ($K_d = 18.9 \pm 3.7$ μM). Preincubation of either HC or LC with 6 mM Ca^{2+} followed by reassociation with the untreated complementary chain in the presence of 0.12 mM Ca^{2+} failed to generate significant cofactor activity (<0.5 nM min^{-1} (nM LC)$^{-1}$). However, pretreatment of both HC and LC with 6 mM Ca^{2+} followed by reassociation (at 0.12 mM Ca^{2+}) generated high activity (7.5 ± 0.4 nM min^{-1} (nM LC)$^{-1}$). Progress curves for activity regain following factor VIII−Ca^{2+} association kinetics fitted well to a series reaction scheme rather than one of simple association ($p < 0.0001$), suggesting a multistep process which may include a Ca^{2+}-dependent conformational change. These results suggest that factor VIII contains two Ca^{2+} binding sites with different affinities and that active factor VIII can be reconstituted from HC and LC only when both chains are preactivated by Ca^{2+}.

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 in subsequent rates of factor Xa generation.

Factor VIII is synthesized as a ~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 a heavy chain (HC[1]) minimally represented by the Al−A2 domains and a light chain (LC) consisting of the A3−C1−C2 domains. Metal ions play an important role in regulating factor VIII structure and activity. The A domains of factor VIII share homology with the A domains of factor V and the copper-binding protein, ceruloplasmin (*9*). One mole of copper has been identified in factor VIII (*10*, *11*). While high (mM) concentrations of Cu^{2+} fail to support factor VIII reconstitution (*12*), low (μM) levels of Cu^{+} or Cu^{2+} stimulate reconstitution in the presence of Ca^{2+} or Mn^{2+} (*13*).

Factor VIII is inactivated by EDTA, which facilitates dissociation of the HC and LC (*6*, *8*). Factor VIII can be reconstituted by combining the isolated subunits in the presence of Ca^{2+} or Mn^{2+} (*12*, *14*, *15*). Thus, it was thought that the linkage of HC and LC by a metal ion (Ca^{2+}, Mn^{2+}, or Cu^{2+}) formed an active heterodimer. In a recent study, we evaluated metal ion-dependent and independent association of factor VIII chains (*16*). In the absence of metal ion, LC and HC combine to form an inactive heterodimer as demonstrated by fluorescence energy transfer. Ca^{2+} has little effect on intersubunit affinity yet converts the inactive dimer to an active, although low 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 high intersubunit affinity and yields a high specific activity factor VIII.

Carboxyl oxygens from acidic amino acid side chains (Glu/Asp) and carbonyl oxygens from peptide bonds are usually involved in Ca^{2+}coordination (*17*). Examples of such Ca^{2+} coordination can be seen in many coagulation factors and include the Gla and EGF domains of many of the vitamin K-dependent serine proteases (*18*). Such Ca^{2+} binding usually results in the stabilization of the Ca^{2+} coordinating loop. Factor V was shown to have a Ca^{2+} binding site with high

† This work was supported by NIH Grants HL 38199 and HL 30616. An account of this study was presented at the 43rd meeting of the American Society of Hematology, December 7, 2001, Orlando, FL.

* Corresponding author. Tel: 716-275-6576. Fax: 716-473-4314. E-mail: philip_fay@urmc.rochester.edu.

[1] Abbreviations: HC, factor VIII heavy chain; LC, factor VIII light chain; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; EDTA, ethylenediamine tetraacetic 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; and FGFR-1, fibroblast growth factor receptor.

10.1021/bi025589o CCC: $22.00
Published on Web 06/05/2002

affinity ($K_d = 24\ \mu M$) (*19*), and the Ca^{2+} in factor V appears to be directly involved in the metal ion linkage between HC and LC (*19*−*21*).

Despite the importance of Ca^{2+} for factor VIII activity, the interactions of factor VIII with Ca^{2+} and mechanisms by which activity is modulated remain largely unknown. In this report, we demonstrate that there are two Ca^{2+} binding sites in factor VIII and that Ca^{2+} must be bound to both HC and LC in order to generate active factor VIII. Our results also suggest that the activity generation due to Ca^{2+} binding to factor VIII is a slow process in which a conformational change is possibly involved.

MATERIALS AND METHODS

Reagents. Recombinant factor VIII preparations (Kogenate) were a gift from Dr. Lisa Regan of Bayer Corporation (Berkeley, CA). The material is stable for >72 h following reconstitution of the lyophilized powder with sterile water. Purified recombinant factor VIII was also a generous gift from Debra Pittman of the Genetics Institute (Cambridge, MA). Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using octylglucoside as described previously (*22*). The reagents α-thrombin, factor IXaβ, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin and phospholipids (Sigma, St. Louis, MO), the chromogenic Xa substrate S-2765 (N-α-benzyloxycarbonyl-D-arginyl-L-glycyl-l-arginyl-*p*-nitroanilide-dihydrochloride; DiaPharma, West Chester, OH), and ^{45}Ca (Amersham-Pharmacia Biotech, Piscataway, NJ) were purchased from the indicated vendors.

Preparation of Factor VIII and Subunits. Factor VIII (Kogenate) was dissolved in 20 mM HEPES, 0.3 M KCl, 0.01% Tween-20, pH 7.2, further concentrated using a CentriPlus concentrator (Millipore, Bedford, MA), dialyzed into the same buffer, and stored at −80 °C. Factor VIII LC and HC were isolated from factor VIII as described (*23*) and were dialyzed into 10 mM MES, 0.3 M KCl, 0.01% Tween-20, pH 6.5, and stored at −80 °C.

Factor VIII Activity Titration by Ca^{2+}. The Ca^{2+}−EGTA buffer with free Ca^{2+} concentrations between 1 μM and 20 mM were made as described (*24*). Briefly, 5 mM EGTA was mixed with Ca^{2+} (1−25 mM) in 10 mM MES, 0.3 M KCl, 0.01% Tween-20, 0.01% BSA, and the pH of each solution was readjusted to 6.5. The free Ca^{2+} concentration was calculated after the adjustment for temperature (23 °C) and salt concentration (0.3 M). HC (100 nM) and LC (100 nM) were associated in each Ca^{2+}−EGTA solution at 23 °C for 18 h, and the activity was measured using the factor Xa generation assay as described below. Nonlinear least squares regression analysis was performed according to a single-site binding model and a two-site binding model (nonidentical and independent sites) using the formula

$$\text{Activity} = \frac{A\cdot[Ca^{2+}]}{K_d + [Ca^{2+}]}$$

and

$$\text{Activity} = \frac{A_{max\,1}\cdot[Ca^{2+}]}{K_{d1} + [Ca^{2+}]} + \frac{A_{max\,2}\cdot[Ca^{2+}]}{K_{d2} + [Ca^{2+}]}$$

where A, A_{max1}, and A_{max2} are constants reflecting maximal activity and K_d, K_{d1}, and K_{d2} are the dissociation constants.

Preincubation of Factor VIII Subunits with Ca^{2+}. HC and LC (4 μM each in 10 mM MES, 0.3 M KCl, 0.01% Tween-20, 0.01% BSA, pH 6.5) were separately preincubated with 6 mM Ca^{2+} or 0.1 mM EDTA for 1 h at 23 °C. Prolonged treatment under these conditions was avoided because of HC instability. Reactions were initiated by mixing HC and LC solutions at a final subunit concentration of 40 nM (residual Ca^{2+} and EDTA concentrations were 0.12 mM and 1 μM, respectively). At the indicated times, aliquots were removed, and the activity was measured by the factor Xa generation assay as described below.

Factor VIII−Ca^{2+} Association Kinetics. HC and LC (4 μM each in 10 mM MES, 0.3 M KCl, 0.01% Tween-20, 0.01% BSA, pH 6.5) were reassociated in the absence of metal ion for 2 h at 23 °C. After addition of 1 mM Ca^{2+}, aliquots were taken at the indicated times, and activity was measured over a time course using the factor Xa generation assay described below. Curve fitting was performed according to model A using the formula below:

$$\text{A: FVIII} + Ca^{2+} \xrightarrow{k} \text{FVIII}{-}Ca^{2+}$$

$$\text{Activity} = A_0\cdot(1 - e^{-kt}), \qquad ([\text{FVIII}] \ll [Ca^{2+}])$$

where A_0 is a constant reflecting maximal activity and k is the observed association rate constant. Additional curve fitting was performed according to model B using the formula below:

$$\text{B: pFVIII} + Ca^{2+} \xrightarrow{k_1} \text{pFVIII}{-}Ca^{2+} \xrightarrow{k_2} \text{aFVIII}{-}Ca^{2+}$$

where pFVIII refers to factor VIII prior to Ca^{2+} treatment (preactivated form) and aFVIII refers to the Ca^{2+}-activated form of factor VIII and

$$\text{Activity} = A_0\cdot\left[1 - \frac{1}{k_2 - k_1}\cdot(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right], \qquad ([\text{FVIII}] \ll [Ca^{2+}])$$

where A_0 is a constant reflecting maximal activity and k_1 and k_2 are the observed association rate constants.

Factor Xa Generation Assays. The rate of conversion of factor X to factor Xa was monitored in a purified system (*25*). Each sample that contained factor VIII or subunits was first 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 0.01% BSA and 10 μM PSPCPE vesicles for 1 min. The reaction was stopped by adding 10 U/ml hirudin, and the resultant factor VIIIa was reacted with factor IXa (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 23 °C. The activity was expressed as the amount of factor

Xa generated (nM) per minute and converted to the value per nM LC.

Equilibrium Dialysis using ^{45}Ca. Equilibrium dialysis was performed as described elsewhere (26, 27). Double distilled H_2O (ddH_2O) was further treated with application to a Chelex 100 column (Bio-Rad, Hercules, CA) and used for the preparation of the reagents. All equipment was washed with chelex 100-treated ddH_2O. The apparatus for equilibrium dialysis (Dialysis Cells, Bel-Art products, Pequannock, NJ) was washed with 10 mM EDTA, followed by an extensive wash with Chelex 100-treated ddH_2O. The equilibrium dialysis apparatus was composed of 2 chambers (volume capacity = 100 μL), which were separated by a dialysis membrane (a molecular weight cutoff = 6000 was employed). Recombinant factor VIII (Kogenate) in 20 mM HEPES, pH 7.2, 0.3 M KCl, 0.01% Tween-20 was concentrated using CentriPlus (Millipore; molecular weight cutoff = 10 000) and was placed in one side of the equilibrium dialysis apparatus in the presence of the indicated amount of Ca^{2+} containing ~2000 Bq ^{45}Ca. Fresh ^{45}Ca (<3 weeks since synthesis) was used to minimize the presence of the decay product, $^{45}Sc^{3+}$, which demonstrates high affinity for many proteins thereby resulting in a reduction of the sensitivity (*28*). After a 48 h incubation at 23 °C, the radioactivity from ^{45}Ca was measured by liquid scintillation counting, and the amounts of free Ca^{2+} and factor VIII-bound Ca^{2+} were calculated. At the salt concentration and pH (slightly higher than the calculated *p*I values of 6.95 and 6.24 for full length and B-domainless factor VIII, respectively) employed, the correction due to the Donnan effect was unnecessary. Nonlinear least-squares regression was performed using the formula

$$\nu = \frac{n \cdot [Ca^{2+}]}{K_d + [Ca^{2+}]}$$

where ν is the ratio of bound $[Ca^{2+}]$ per [factor VIII] and n is the number of binding sites.

Direct Measurement of ^{45}Ca Binding to Factor VIII and Subunits. Chelex 100-treated ddH_2O was used for the preparation of the reagents and to wash all equipment. Intact factor VIII, HC, HC plus LC, or LC (each at 9 μM) in 200 μL 20 mM HEPES, pH 7.2, 0.3 M KCl, 0.01% Tween-20 was mixed with ~100 000 Bq ^{45}Ca (40 μM) for 18 h at 23 °C. A negative control contained buffer alone. The samples were dialyzed against 200 mL 20 mM HEPES, pH 7.2, 0.3 M KCl, 0.01% Tween-20 for 6 h at 23 °C with one buffer change. Aliquots were removed, and the remaining ^{45}Ca was measured by liquid scintillation counting. The values of samples were subtracted by the value of negative control and normalized as (scintillation) counts per mol protein (cpm/μM).

Statistical Analysis. Nonlinear least squares regression analysis was performed using Kaleidagraph (Synergy, Reading, PA), and the parameters and their standard errors were obtained. The best model to fit to the data was determined by an *F*-test comparing the sum of squares from each fitting. The degrees of freedom in each data set was obtained by subtracting number of parameters from the number of data points. The percentage points (probability) for the *F*-distribution were calculated using Microsoft Excel.

RESULTS

Equilibrium Ca^{2+} Binding to Factor VIII Detected by Functional Assay. Ca^{2+} binding to factor VIII was monitored by measuring the regeneration of factor VIII cofactor activity for factor IXa since factor VIII reconstituted from its component HC and LC shows little if any activity in the absence of this metal ion (*16*). Thus, titration of Ca^{2+} was used to assess the affinity of this ligand for factor VIII. Initial experiments indicated that the reassociation of HC and LC in the presence of low (μM) concentrations of Ca^{2+} generated significant activity after prolonged incubation (several days, results not shown), suggesting the existence of high-affinity Ca^{2+} binding site(s) in factor VIII. Difficulty in controlling low Ca^{2+} concentrations in the reaction solution prompted us to use a Ca^{2+}−EGTA buffer system in order to maintain low levels of free Ca^{2+} concentration. Furthermore, the presence of 5 mM EGTA in this system was useful to eliminate the effect of any other contaminating metal ions that may affect factor VIII activity.

One potential concern with using this buffer system was the possibility of binding of EGTA to factor VIII with subsequent effect on cofactor activity. The basis for this consideration is the reported nonspecific binding of EDTA to BSA through histidine residues (*29*). However, the following experiment suggested that any binding of the chelator to factor VIII did not of itself affect factor VIII activity. When 1 μM factor VIII was treated with 5 μM EGTA, >95% of the activity was lost after an 18 h incubation (data not shown). Assuming EGTA would bind factor VIII with similar affinity as that of EDTA for BSA (K_d ~0.4 mM; (*29*)), <2% of the factor VIII would have associated the chelator, given the above reactant concentrations. Furthermore, inclusion of either BSA or the Ca^{2+}-binding protein, troponin C at 50 μM in the above reaction yielded equivalent inhibition of factor VIII by EGTA (results not shown), suggesting that these potential competitors for EGTA binding showed no effect in the chelator-dependent inhibition of cofactor activity. Taken together, these results indicated that the basis for EGTA-mediated inhibition of factor VIII was by metal ion chelation rather than direct binding of the chelator to the factor VIII protein.

Results from titrating Ca^{2+} in factor VIII reconstitution using the functional assay are presented in Figure 1. For this analysis, factor VIII HC and LC were employed at 100 nM (~2 times Kd, *16*), precluding the need for Cu^{2+} in enhancing the interchain affinity, since the presence of this metal ion could be perturbed by inclusion of EGTA. Reconstituted factor VIII was diluted 20-fold in the factor Xa generation assay and activity determined immediately. Control experiments showed no effect of the EGTA levels employed on the rates of factor Xa generated. As shown in Figure 1, the binding curve was well fitted by a two-site binding model as compared with a single-site binding model. Statistical analysis by *F*-test indicated that the reduction of sum-of-squares was highly significant ($P < 0.0001$) when the data were fitted by the two-site binding model. Furthermore, the reduction of sum-of-squares was not significant ($P = 0.573$) when the data were fitted by a three-site binding model compared with that of the two-site model (*F* value = 0.888, data not shown). These results indicate that factor VIII reconstituted from isolated subunits generates maximal

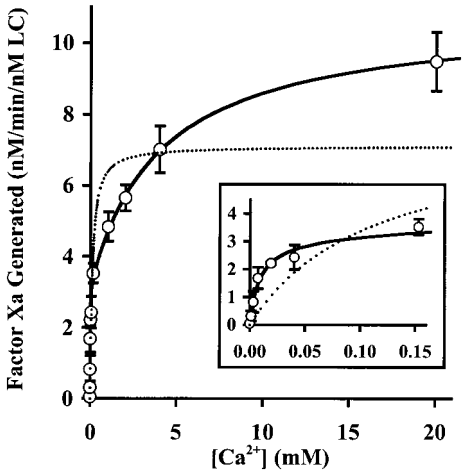

FIGURE 1: Equilibrium binding of Ca^{2+} to reconstituted factor VIII assessed by a functional assay. Mixtures of 100 nM LC, 100 nM HC, and the indicated amounts of Ca^{2+} were incubated for 18 h at 23 °C, and reconstituted factor VIII activity was measured by the factor Xa generation assay as described in Materials and Methods. Each point represents the average value of quadruplicated samples. Lines were drawn from the curve fits according to a single-site binding model (dotted line) and a two-site binding model (solid line) as described in Materials and Methods. The inset displays the data for the lower Ca^{2+} concentrations.

cofactor activity only following Ca^{2+} binding to both the high-affinity (K_d = 8.9 μM) and the low-affinity sites (K_d = 4.0 mM) listed in Table 1.

Ca^{2+} Binding to Factor VIII as Detected by Equilibrium Dialysis. The binding of Ca^{2+} to factor VIII was determined by equilibrium dialysis using ^{45}Ca as a probe. This analysis utilized intact factor VIII that was not treated initially with chelators (see below). Thus, in this experiment, we measured the exchange of Ca^{2+} for the radioactive isotope. For this reason, an extended reaction time of 48 h was employed. One limitation to this method was the available concentration of factor VIII (maximally 13.5 μM) for analysis. Following reaction of factor VIII with ^{45}Ca, differences of <10% in measurable radioactivity for the factor VIII-containing and factor VIII-lacking chambers of the equilibrium dialysis apparatus were difficult to quantify, given the ~5% variation in scintillation counting. Therefore, limitation in factor VIII concentration precluded determination of a lower-affinity Ca^{2+} binding site (K_d > ~ 0.1 mM). However, these concentrations in reagent were amenable to evaluate a high-affinity Ca^{2+} binding site. Results from this analysis are shown in Figure 2, where the ^{45}Ca titration was performed up to 0.4 mM. These data fitted well with the single-site binding model up to the limits of the free Ca^{2+} concentration employed (R^2 = 0.96) and yielded a high-affinity Ca^{2+} binding site in factor VIII (K_d = 18.9 ± 3.65 μM; Table 1). This value was in close agreement with the high-affinity value determined using the functional assay described above.

A binding stoichiometry derived from the fitted curve was 1:0.79 (factor VIII:Ca^{2+}), indicating little less than 1 mol Ca^{2+} bound per mol factor VIII. The reason that this value was less than unity is not fully understood. In a separate experiment, a significant reduction in ^{45}Ca binding (as much as 40%) was observed when factor VIII was stored for several days at 4 °C (data not shown). Thus, factor VIII may demonstrate some instablity in maintaining Ca^{2+} binding capability or Ca^{2+} exchange efficiency under these conditions. Since the duration of the equilibrium dialysis analysis (48 h) was necessary for the complete association of ^{45}Ca, this instability of factor VIII may account for the value obtained in binding stoichiometry. Intact factor VIII untreated with chelators was employed in these studies in order to use material possessing maximum cofactor activity, based upon an observed reduction in the activity of factor VIII reconstituted from subunits that are prepared following chelation of factor VIII when compared with native factor VIII activity (results not shown). Furthermore, no significant binding of ^{45}Ca was observed following equilibrium dialysis using isolated factor VIII HC or LC (data not shown). The reasons for reduced binding of Ca^{2+} to the EDTA-treated factor VIII and subunits are unclear. Alternatives may include a weakened affinity for the metal ion resulting from a conformational instability following initial extraction of the originally bound ligand and/or reflect a reduced population of active factor VIII chains following this treatment.

Applying the K_d value (4030 μM) for the low-affinity site, obtained from Figure 1, we performed curve fitting by the formula below:

$$\nu = \frac{n_1 \cdot [Ca^{2+}]}{K_d + [Ca^{2+}]} + \frac{n_2 \cdot [Ca^{2+}]}{4030 + [Ca^{2+}]},$$

where ν is the ratio of the bound [Ca^{2+}] per [factor VIII] and n_1 and n_2 are the number of respective binding sites on factor VIII. Although after performing an *F* test we obtained no significant improvement compared to the single-site binding model (due to the lack of the data points at the high Ca^{2+} concentration), this fitting yielded a reasonable result (sum of squares = 0.0367, R^2 = 0.965). Therefore, this exercise suggested that this binding data would fit well with the data points for higher free [Ca^{2+}], if available, in describing a low-affinity binding site (K_d ≥ mM), consistent with that obtained from the factor Xa generation assay.

Ca^{2+} Binding to Individual Subunits Detected by Ca^{2+} Pretreatment of Factor VIII Subunits and Activity Reconstitution. Results described above demonstrate Ca^{2+} binding to factor VIII using two independent approaches. As indicated in the above experiment, we could not obtain quantitative data on Ca^{2+} binding to the isolated factor VIII chains using equilibrium dialysis. However, an attempt was made to further clarify and localize the Ca^{2+} binding site in factor VIII using the functional assay. For this analysis, isolated subunits were treated with either Ca^{2+} (6 mM) or EDTA (0.1 mM) prior to recombining the subunits. Cofactor activity was then determined using a factor Xa generation assay for a time course following mixing of the factor VIII chains as described in Methods. Only when both HC and LC were pretreated with $Ca^{2+}$was significant active factor VIII generated (Figure 3, open circles). At the time of HC and LC reassociation, residual Ca^{2+} and EDTA concentrations were 0.12 mM and 1 μM, respectively. This level of Ca^{2+} caused minimal increases in activity of the samples (Figure 3, triangles and squares) as compared with the absence of Ca^{2+} (Figure 3, diamonds). Therefore, the pronounced activity generated after mixing the Ca^{2+}-pretreated HC and LC was likely the result of a Ca^{2+}-dependent alteration in the subunits, and this suggested that both HC and LC were separately affected following interaction with Ca^{2+}.

Table 1: Parameters for Factor VIII and Ca^{2+} Binding[a]

equilibrium parameters	K_d (μM)	*F* value	*P* value
factor Xa generation assay			
single-site binding model	109 ± 62.4		
two-site binding model	8.9 ± 1.85 and 4030 ± 623	65.0	<0.0001
^{45}Ca equilibrium dialysis			
single-site binding model	18.9 ± 3.65		
kinetic parameters	k_1[b] × 1000	k_2[b] × 1000	*F* value
simple association	2.44 ± 0.28	–	
series reaction	5.39 ± 1.46	10.2 ± 3.57	5.16

[a] Parameter values were calculated by nonlinear least-squares regression on the data shown in Figures 1, 2, and 4 using the formula shown in Materials and Methods. [b] Association rate constant, observed (min^{-1}).

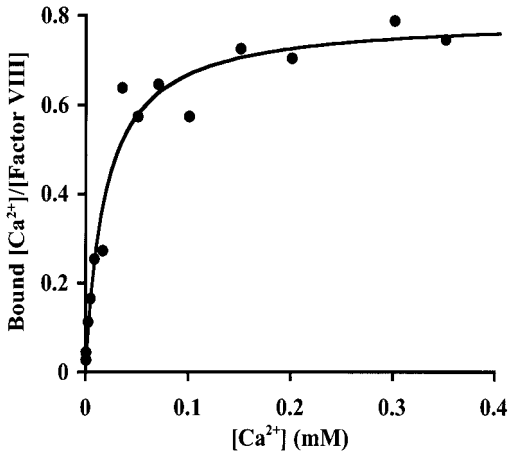

FIGURE 2: Binding of Ca^{2+} to factor VIII determined by equilibrium dialysis. The amount of Ca^{2+} bound to factor VIII (13.5 μM) was measured after 48 h incubation in the presence of the indicated amount of free Ca^{2+} containing ~2000 Bq ^{45}Ca by equilibrium dialysis as described in Materials and Methods. Each point represents the average value of three independent experiments. A line was drawn by the curve fitting according to the model as described in Materials and Methods.

Ca^{2+} Binding to Individual Subunits Detected by Direct ^{45}Ca Binding Measurement. In an alternate attempt to gain insights into the interaction of Ca^{2+} with isolated factor VIII chains, we attempted to directly measure ^{45}Ca bound to the subunits. This method is semiquantitative but demonstrates high sensitivity due to negligible background signal from the unbound ^{45}Ca in the sample solution (*28*). After ^{45}Ca binding to equivalent concentrations of factor VIII, reassociated HC/LC, HC, or LC reached equilibrium, the samples were dialyzed to remove unbound ^{45}Ca and the residual radioisotope bound to the samples was counted by liquid scintillation. Thus, the value obtained would correspond to the amount of bound ^{45}Ca at a point in the dissociation process rather than represent an equilibrium value. Each obtained value was adjusted for the negative control (no protein sample). While the low levels of Ca^{2+} incorporation preclude rigorous conclusions, the results of this analysis, shown in Table 2, demonstrate that intact factor VIII, which was not treated with chelators, bound ^{45}Ca several-fold greater than the reassociated subunits. The reason for this disparity is not known but may reflect instability of the Ca^{2+} sites in the chelator-treated chains. HC also demonstrated a capacity to bind ^{45}Ca that was fractional compared to that observed for the HC/LC complex. No detectable binding was observed for the isolated factor VIII LC by this method.

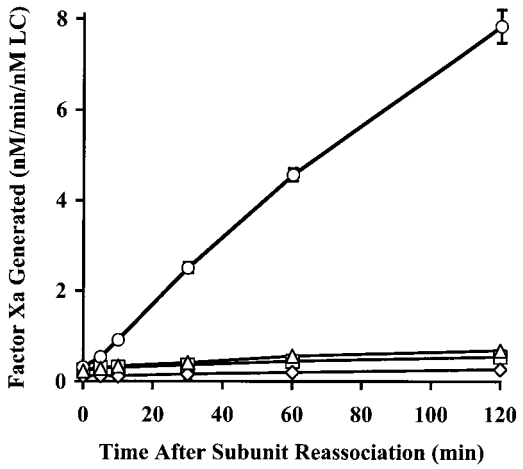

FIGURE 3: Effect of preincubation with Ca^{2+} on factor VIII reconstitution from isolated subunits. Factor VIII subunits (HC or LC) were preincubated with 6 mM Ca^{2+} or 0.1 mM EDTA for 1 h. After the preincubated HC and LC was mixed, reconstituted factor VIII activity was measured by the factor Xa generation assay as described in Materials and Methods. The combinations of the mixture were HC preincubated with Ca^{2+} plus LC preincubated with Ca^{2+} (open circles), HC preincubated with EDTA plus LC preincubated with Ca^{2+} (squares), HC preincubated with Ca^{2+} plus LC preincubated with EDTA (triangles), and HC preincubated with EDTA plus LC preincubated with EDTA (diamonds). Each point represents the average value of quadruplicated samples.

Table 2: Direct Measurement of ^{45}Ca Binding to Factor VIII and Its Subunits[a]

factor VIII or subunits	bound ^{45}Ca × 10^3 (mol/mol factor VIII)
HC	0.43 ± 0.02
LC	0 ± 0
HC/LC	1.28 ± 0.054
intact factor VIII[b]	9.5 ± 0.041

[a] Factor VIII or subunits (9 μM each) were incubated with ^{45}Ca (~100 000 Bq, 40 μM) for 24 h. The samples were dialyzed for 6 h to remove unbound ^{45}Ca, and the bound ^{45}Ca was determined by liquid scintillation counting as described in Materials and Methods. [b] Factor VIII untreated with chelators.

Factor VIII Activity Reconstitution Kinetics Mediated by Ca^{2+} Binding. To evaluate the rate of Ca^{2+}-mediated regain in factor VIII activity reconstituted from isolated chains, we first associated high concentrations (4 μM) of HC and LC in the absence of metal ion for 2 h. This reaction time was obtained from a parallel experiment using acrylodan-labeled LC (fluorescence donor) and fluorescein-labeled HC (fluorescence acceptor), where the association kinetics were monitored by fluorescence resonance energy transfer using

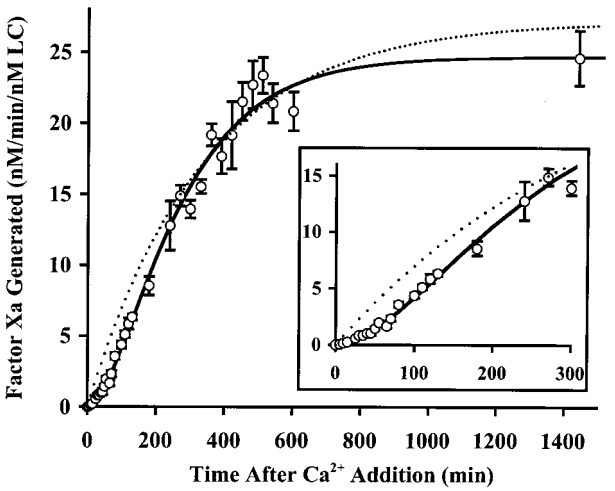

FIGURE 4: Factor VIII activity generation kinetics as a result of Ca^{2+} association. HC and LC (4 μM each) were reassociated in the absence of metal ion for 2 h. After addition of 1 mM Ca^{2+}, the activity was measured over a time course by the factor Xa generation assay as described in Materials and Methods. Each point represents the average value of quadruplicated samples. A line was drawn from curve fitting according to either the simple reaction model (dotted line) or series reaction model (solid line) as described in Materials and Methods. The inset displays the data from the earlier time points.

our previously described methods (*16*). Those results (not shown) indicated that HC and LC association, as detected by the quenching of acrylodan fluorescence, reached a maximal value within 1 h, consistent with a high level of heterodimer formation. However, a fraction of the factor VIII chains appeared refractory to forming the dimer. Approximately 50% of the acrylodan-labeled LC associated with HC using a 4-fold excess of the latter, as judged by gel filtration analysis. This result was independent of the presence of Ca^{2+} (results not shown). This observation was consistent with our past reports for the specific activity of the reconstituted HC and LC representing ~30% that of intact factor VIII (*12*, *16*).

Subsequently, the time-dependent generation of factor VIII cofactor activity was monitored following Ca^{2+} addition (Figure 4). Thus, the activity observed in this experiment should result from the association of Ca^{2+} with the metal ion-independent factor VIII dimer. Since the association of two reactants typically follows second-order kinetics, the rate of reassociation is proportional to the product of the concentration of free reactants. Since at time 0 the concentrations of both the free reactants are maximal, the velocity of reassociation as represented by the slope of the curve should be highest for the initial time points. However, as noted in the figure, the slope of the curve demonstrates a lag phase extending to ~100 min. This lag would occur if additional reaction steps were required after the association reaction between factor VIII and Ca^{2+} and before generation of the active factor VIII molecule. The data fit well with such a series reaction model as judged by the significant reduction of the sum of squares as compared to the sum of squares fitted by the simple association model ($P < 0.0001$, Table 1).

DISCUSSION

The binding of Ca^{2+} to factor VIII is critical for the generation of cofactor activity. In the current study, we examined this association using a combination approach employing both functional and physical assays. Results obtained from factor Xa generation assays indicated an interaction that was reasonably fitted by a two-site binding model and yielded K_d values of 8.9 μM and 4.0 mM. A high-affinity Ca^{2+} binding site ($K_d = 18.9$ μM) was verified using equilibrium dialysis. However, limitations in the available concentrations of factor VIII and sensitivity of this method precluded identification of low-affinity site(s). Examination of the isolated factor VIII chains proved problematic in evaluating the association of Ca^{2+}. However, functional studies using factor VIII chains that had been preincubated with the metal ion prior to reconstitution of activity yielded suggestive evidence that Ca^{2+} bound sites on both the HC and LC and that occupancy of these sites was required for cofactor function. Additional support for a Ca^{2+} site on the HC was obtained following analysis of direct binding assays employing the radioisotope.

Earlier studies by Guinto and Esmon (*19*) demonstrated that factor Va, a homologous protein to factor VIII, possessed a high-affinity Ca^{2+} binding site ($K_d = 24$ μM) when either intact factor Va or factor Va reassociated from its subunits was examined. In addition, these investigators showed that intact factor Va appeared to possess additional, low-affinity Ca^{2+} binding sites ($K_d \sim 1-4$ mM). The corresponding high and low-affinity sites on factor Va are analogous to the high- and low-affinity sites we found on factor VIII ($K_d = 8.9-18.9$ μM and ~4 mM, respectively). However, several differences in the interactions of Ca^{2+} with the proteins/subunits appear to exist. Although the individual subunits (HC or LC) of factor Va did not bind Ca^{2+}, the reassociated HC and LC recovered full Ca^{2+} binding capability (*19*). On the other hand, our data suggest that Ca^{2+} binds to both HC and LC of factor VIII and this binding of Ca^{2+} to each subunit is critical in generating active factor VIII. For reasons that are not well understood, isolated factor VIII chains fail to fully regain activity upon reassociation, typically yielding ~30% the specific activity of native factor VIII (*16*). This limitation may reflect a conformational instability following initial removal of the metal ion. Several lines of evidence support this possibility including failure of excess HC to completely saturate LC independent of the presence of Ca^{2+}, as judged by gel filtration analysis, as well as limited success in detecting Ca^{2+} binding to the isolated subunits by direct or equilibrium dialysis methods. Furthermore, Ca^{2+} regulates HC and LC association on factor Va in that its presence dramatically increases the interchain binding affinity (*19*, *20*). Thus, Ca^{2+} is likely coordinated between HC and LC in factor Va. Alternatively, a recent study assessing the association of factor VIII HC and LC using fluorescence energy transfer showed no Ca^{2+}-dependent effect on the interchain affinity (*16*).

Ca^{2+} interaction with factor VIII HC was suggested by direct measurement of ^{45}Ca binding. However, no evidence for direct binding of the metal ion to LC was observed. This latter result contradicted the functional data obtained following preincubation of the isolated chains with Ca^{2+}, which indicated that reaction of Ca^{2+} with both chains was required for regain of cofactor activity. Thus, failure of LC to react with a low concentration of ^{45}Ca (40 μM) may reflect reduced affinity at this site. On the basis of the available data, we hypothesize that the low-affinity (K_d ~4 mM) Ca^{2+} binding

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Factor V     94LSEGASYLDHTFPAEKM
Factor VIII 108ASEGAEYDDQTSQREKE
FGFR1       124SSEDDDDDDDSSSEEKE
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FIGURE 5: Sequence alignments of factor V, factor VIII, and FGFR-1. Residues are indicated by the single letter designation. Acidic residues are in bold typeface.

site is located in the LC while the high-affinity site (K_d = 8.9−18.9 μM) is present in the HC. This speculation is fully compatible with the results obtained from the Ca^{2+} preincubation experiment in which the Ca^{2+} concentration (6 mM) was saturating with respect to the high-affinity site and reflected ~60% saturation relative to the low-affinity site.

Little information is available on sites for Ca^{2+} binding in factor VIII. However, recent studies in the homologous factor V have localized a Ca^{2+} binding site. Using fragments derived from a plasmin digest of factor Va HC, Zeibdawi et al. (*21*) demonstrated that residues 94−110 participated in the Ca^{2+}-dependent association of factor Va HC and LC. This region of the A1 domain contains three acidic residues and is localized adjacent to the A3 domain of LC. Alignment of factor V and VIII sequences reveals that this region corresponds to residues 108−124 in the A1 domain of factor VIII. Comparison of these segments (Figure 5) indicated 53% sequence identity (9/17 residues) with conservation of the acidic residues located in the factor V sequence. Furthermore, this region in factor VIII contains an additional three acidic amino acid residues. Interestingly, a homology search using FASTA3 (*30*, *31*) identified residues 124−140 in fibroblast growth factor receptor (FGFR-1) as possessing 47% sequence identity. This region in FGFR-1, referred to as an acidic box, is reported to represent a Ca^{2+} binding site (*32*).

Comparison of these sequences may explain the observed disparity that isolated factor Va HC fails to bind Ca^{2+} (*19*), whereas the HC of factor VIII does bind the metal. For example, the three acidic residues identified in the factor V 94−110 segment may be insufficient to chelate the Ca^{2+} ion and may require additional ligands contributed from the LC to form this binding site. Thus, Ca^{2+} binding would directly contribute to and augment the interchain affinity for factor Va. Alternatively, the presence of additional acidic residues in factor VIII sequence 108−124 supports the speculation that there may be sufficient ligands in this region for Ca^{2+} coordination in the absence of any contribution from LC residues. Thus Ca^{2+} would not necessarily modulate the interchain affinity in factor VIII.

Interestingly, examination of the kinetics of activity generation following Ca^{2+} binding revealed a lag phase suggestive of a multistep process rather than a mechanism whereby cofactor activity is generated in a direct response to binding the metal ion. We speculate that this lag is the result of a Ca^{2+}-dependent change in conformation which precedes the development of cofactor activity. Thus, the lag period was not apparent following the Ca^{2+} preincubation experiments. Consistent with this speculation are earlier studies from our laboratory showing that treatment of intact factor VIII with EDTA decreased its intrinsic fluorescence, indicating an altered environment surrounding certain tryptophan residues due to conformational change with loss of divalent metal ion (*33*). That study also showed that Ca^{2+} likely binds to a site on the factor VIII HC close to an exposed hydrophobic patch postulated to be near the LC interface. It is interesting to note the sequence VSLHAVGVSYW comprising factor VIII residues 96−106, which is rich in apolar residues and contains a Trp residue as a potential reporter of intrinsic fluorescence, is in close proximity to the putative Ca^{2+}-binding site as suggested above. More recently, fluorescence energy transfer between acrylodan-labeled LC (fluorescence donor) and fluorescein-labeled HC (fluorescence acceptor) indicated a significant difference in energy transfer efficiency when measured in the presence or absence of Ca^{2+} (*16*). This result indicated a Ca^{2+}-dependent alteration in the inter-fluorophore spatial separation. Consistent with these observations are studies examining the intrinsic fluorescence during factor Va subunit reassociation in the presence of Ca^{2+} suggesting that the metal ion elicits modest changes in tertiary and quaternary structure correlating with the regeneration of activity (*34*).

Results presented in this report have identified two Ca^{2+} binding sites with different affinities show that active factor VIII can be reconstituted from HC and LC only when both chains are preactivated with Ca^{2+} and suggest that factor VIII activity reconstitution involves a series of steps, one of which may be a Ca^{2+}-dependent change in conformation. However, the mechanisms by which Ca^{2+} modulates activity of factor VIII remain largely unknown and resolution of this issue may represent a complex scheme. For example, residues 108−124, which we speculate may contain a putative Ca^{2+}-site, reside on a face of the A1 domain (*35*) that is directed away for the factor IXa-interactive sites localized within the A2 (*36*, *37*) and A3 domains (*38*). Therefore, transduction of a Ca^{2+}-dependent interaction in factor VIII to yield a productive interaction of cofactor and enzyme may necessarily occur over a significant distance.

ACKNOWLEDGMENT

We thank Dr. Lisa Regan of the Bayer Corporation and Debbie Pittman the Genetics Institute for the gifts of recombinant human factor VIII. We also thank Drs. Charles W. Francis and Abha Sahni, and Carrie-Ann Ballard for assistance in experiments involving radioisotopes.

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BI025589O