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pH-dependent association of factor VIII chains: Enhancement of affinity at physiological pH by Cu²⁺

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Abstract

Reconstitution of factor VIII from isolated heavy chain (HC) and light chain (LC) shows pH-dependence. In the presence of Ca²⁺, up to 80% of native factor VIII activity was recovered over a wide range of pH. In contrast, affinity of HC and LC was maximal at pH 6.5–6.75 ($K_d \sim 4$ nM), whereas a $K_d \sim 20$ nM was observed at physiological pH (7.25). The effect of Cu²⁺ (0.5 μ M total Cu²⁺) on maximal activity regenerated was negligible at pH 6.25–8.0. However, this level of Cu²⁺ increased the inter-chain affinity by ~ 5 fold at pH 7.25. This effect resulted from an ~ 1.5 fold increased association rate constant (k_{on}) and an ~ 3 fold reduced dissociation rate constant (k_{off}). High affinity ($K_d = 5.3$ fM) of the factor VIII heterodimer for Cu²⁺ was estimated by increases in cofactor activity. No significant increase in inter-chain affinity was observed when either isolated chain was reacted with Cu²⁺ followed by addition of the complementary chain. Together, these results suggest that the protonation state of specific residues modulates inter-chain affinity. Furthermore, copper ion contributes to the maintenance of the heterodimer at physiologic pH by a mechanism consistent with bridging the two chains.

Keywords

factor VIII; copper; factor Xa generation; binding affinity; binding kinetics; pH

1. Introduction

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 reductions in factor IXa activity and subsequent rates of factor Xa generation.

Factor VIII is synthesized as an ~ 300 -kDa single chain precursor protein [3,4] with a domain structure designated as 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 (HC¹) minimally represented by the A1-A2 domains; and a light chain (LC) consisting of the A3-C1-C2 domains.

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Metal ions play an important role in regulating factor VIII structure and activity. Factor VIII is inactivated by EDTA, which facilitates dissociation of the HC and LC [6,8]. Factor VIII activity is regenerated by combining the isolated subunits in the presence of Ca^{2+} or Mn^{2+} [9–11]. In addition, low levels of Cu^+ or Cu^{2+} enhance this effect [12,13]. 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. This interpretation was consistent with studies examining the reconstitution of factor Va from isolated subunits by Ca^{2+} or Mn^{2+} [14,15]. We recently evaluated metal ion-dependent and independent association of factor VIII chains [16]. In the absence of metal ion, LC and HC combined to form an inactive heterodimer as demonstrated by fluorescence energy transfer. Ca^{2+} had little effect on inter-subunit affinity yet converted the inactive dimer to an active one. In contrast, Cu^{2+} significantly enhanced the inter-subunit affinity but yielded a dimer that lacked activity. However, the presence of both metal ions resulted in a high inter-subunit affinity and yielded a high specific activity factor VIII. In addition, Ca^{2+} binding to both factor VIII subunits was required for the generation of cofactor activity [17]. These studies also demonstrated a local conformational change in response to Ca^{2+} binding that correlated with formation of the active cofactor. Recently, mutagenesis studies defined a Ca^{2+} binding site within A1 domain residues 110–126 which partially overlapped with a coordination site for Mn^{2+} [18].

Copper ions appear important for the structural integrity and the function of factor VIII. Based on homology with ceruloplasmin, three Cu^{2+} binding sites have been proposed [19]. 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] and a single consensus type II copper site has been suggested at the A1/A3 domain junction involving His99 in A1 and His1957 in A3 plus oxygen atom of a water molecule H-bonded to Tyr105 and Ala100 [19]. One mol of copper has been identified in factor VIII by electron paramagnetic resonance [12] and atomic absorption spectroscopy [20]. However, the location of the copper ion is not known.

Earlier results have demonstrated that the interaction of A2 subunit with the A1/A3C1C2 was pH-dependent [21,22]. In this study, HC/LC association, which is primarily mediated by interaction of the A1 domain with the LC [23], was evaluated over a range of pH values. Results indicate that inter-chain affinity is enhanced at slightly acidic pH. While the Cu^{2+} -dependence for chain association is largely ameliorated under these conditions, Cu^{2+} exhibits a more significant role in stabilizing the heterodimer at physiological pH.

2. Materials and Methods

2.1. Reagents

Recombinant factor VIII (Kogenate™) was a gift from Dr. Lisa M. Regan of Bayer Corporation (Berkeley, CA). Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE were prepared using octylglucoside as described previously [24]. The reagents α -thrombin, factor IXa β , factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin, phospholipids, and the chromogenic Xa substrate S-2765 (N- α -benzyloxycarbonyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide-dihydrochloride; DiaPharma, West Chester, OH) were purchased from the indicated vendors.

2.2. Preparation of factor VIII and subunits

Factor VIII HC and LC subunits were isolated from factor VIII as previously described [25]. Proteins were dialyzed into 10 mM MES, 0.3 M KCl, 0.01% Tween-20, pH 6.5, and stored at -80°C . Protein concentration was determined by Bradford protein assay [26] using BSA as the standard. Purified LC and HC were >98% and ~95% pure, respectively, according to

density measurements following SDS-PAGE and assay for residual activity by factor Xa generation assay (described below).

2.3. Factor Xa generation assay

The rate of conversion of factor X to factor Xa was monitored in a purified system [27] according to methods previously described [16,17]. Briefly, reconstituted factor VIII samples were diluted (1/20) in buffer containing 20 mM HEPES, pH 7.2, 0.1 M NaCl, 0.01% Tween 20, 0.01% BSA, 5 mM CaCl₂, and 10 μM PSCPE vesicles, and activated with 1 nM α-thrombin for 1 min. The reaction was stopped by adding 5 U/ml hirudin and the resultant factor VIIIa was reacted with factor IXa (40 nM) for 1 min. Factor X substrate (300 nM) was added to initiate the reaction and after 1 min, the reaction was quenched by addition of 50 mM EDTA. Amounts of factor Xa generated were determined following reaction with the chromogenic substrate, S-2765 (0.46 mM final concentration). All reactions were run at 23°C. The activity is expressed as the amount of factor Xa generated (nM) per minute and converted to the value per nM factor VIII subunit.

2.4. HC/LC binding affinity

HC (10 nM) was incubated with LC (0–300 nM) for 6 hours at 23°C in buffer containing 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, and 25 mM CaCl₂ at pH 6.0 - 8.0 in the absence or presence of 0.5 μM Cu²⁺. Reconstitution of factor VIII was performed at moderate ionic strength based upon earlier studies [9,10] demonstrating this condition was optimal for chain association. Buffer solutions at pH 6.0 - 6.5, 6.75, or 7.0 - 8.0 were composed of MES, MOPS, or HEPES, respectively (20 mM each). Following incubation at the indicated pH the samples were diluted into pH = 7.2 buffer. Factor VIII activity was measured by factor Xa generation assay as a function of LC concentration. The presence of 25 mM free Ca²⁺ ensured maximal activity once heterodimers were formed [16, 17]. Data points were fitted to a quadratic equation by non-linear least squares regression using the equation,

$$A = k \times \left(L_0 + H_0 + K_d - \sqrt{(L_0 + H_0 + K_d)^2 - 4 \times L_0 \times H_0} \right) \quad (1)$$

$$V_{\max} = 2 \times k \times H_0 \quad (2)$$

where A is activity (nM/min/nM HC), H_0 is the added HC concentration (10 nM), L_0 is the added LC concentration (0–300 nM), K_d is the dissociation constant, k is a constant to convert concentration to activity, and $V_{\max}$ is activity when HC is saturated by LC.

2.5. HC/LC association rate measurement in the absence or presence of Cu²⁺

HC (10 nM) or LC (200 nM) was separately incubated for 4 hours at 23°C in HEPES reconstitution buffer in the absence or presence of 0.5 μM Cu²⁺. Reactions were initiated by mixing the solutions and aliquots were removed from 1 to 34 min. Factor VIII activity as a function of reassociation time was determined by factor Xa generation assay and data points were fitted to a single exponential curve by non-linear least square regression using the equation,

$$A = A_0 \times \left(1 - e^{-k_{ob} \cdot t} \right) \quad (3)$$

where A is activity (nM/min/nM HC), A_0 is the maximum activity when equilibrium is established, k_{ob} is the observed association rate constant, and t is the time in minutes after reactants were mixed. Subsequently, the association rate constant (k_{on}) was calculated by the following equation.

$$k_{on} = \frac{k_{ob}}{(L_0 + K_d)} \quad (4)$$

where L_0 is LC concentration which is approximated by the initial LC concentration (200 nM), and K_d is the dissociation constant at pH 7.25.

2.6. Measurement of Cu^{2+} affinity for factor VIII

Initially, Cu^{2+} - Ca^{2+} -EGTA buffers with free Cu^{2+} concentrations ranging from 0–2.8 pM (total Cu range 0–90 μM), in the presence of 25 mM Ca^{2+} and 0.2 mM EGTA were prepared based upon the apparent K_d values for the Cu^{2+} -EGTA and Ca^{2+} -EGTA complexes, which in 20 mM HEPES, 0.3 M KCl, pH 7.25 were determined as 22.3 fM and 159 nM, respectively, according to the WinMaxC program (www.stanford.edu/~cpatton/maxc.html). Chelex-100 (Bio-rad) treated ddH₂O was used for buffer preparation to minimize the contamination of metal ions. The free EGTA concentration was then obtained by solving the below equation by the Newton-Raphson method.

$$t\text{EGTA} - [\text{EGTA}] - \frac{t\text{Cu} \cdot [\text{EGTA}]}{[\text{EGTA}] + K_{\text{Cu}}} - \frac{t\text{Ca} \cdot [\text{EGTA}]}{[\text{EGTA}] + K_{\text{Ca}}} = 0 \quad (5)$$

where $t\text{EGTA}$ is the total EGTA, $t\text{Cu}$ is the total Cu^{2+} , $t\text{Ca}$ is the total Ca^{2+} , $[\text{EGTA}]$ is the free EGTA concentration, K_{Cu} is the dissociation constant of Cu^{2+} and EGTA, and K_{Ca} is the dissociation constant of Ca^{2+} and EGTA. Subsequently, free Cu^{2+} concentrations were calculated for the reactions with total Cu^{2+} added ranging from 0–0.09 mM. HC (10 nM) and LC (15 nM) were incubated in the indicated Cu^{2+} - Ca^{2+} -EGTA buffer at 23°C for 6 hours and resultant factor VIII activity was measured using the factor Xa generation assay. Non-linear least squares regression analysis was performed according to a single-site binding model using the formula,

$$\text{Activity} = C + \frac{A_{\text{max}} \cdot [\text{Cu}^{2+}]}{K_{d1} + [\text{Cu}^{2+}]} - \frac{B_{\text{max}} \cdot [\text{Cu}^{2+}]}{K_{d2} + [\text{Cu}^{2+}]} \quad (6)$$

where A_{max} is the maximum incremental increase of activity when Cu^{2+} saturates the high affinity site, $[\text{Cu}^{2+}]$ is free Cu^{2+} concentration, K_{d1} is the dissociation constant for the high affinity site, C is the activity in the absence of Cu^{2+} , B_{max} is the maximum incremental decrease of activity when Cu^{2+} saturates the low affinity site, and K_{d2} is the dissociation constant for the low affinity site.

2.7. Cu^{2+} preincubation of isolated chains

Cu^{2+} (1 μM) was added to HC (1 μM) or LC (1 μM) in 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, pH 7.25 for 1 hour, followed by dialysis into 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, pH 7.25 (The volume ratio of dialysis was 1:20,000). Assuming buffer exchange was complete after dialysis, free Cu^{2+} concentration was ~50 pM. HC or Cu^{2+} -treated HC (10 nM, 1:100 dilution of the sample with the buffer) was incubated with 0–80 nM LC. Alternatively, LC or Cu^{2+} -treated LC (10 nM) was incubated with 0–80 nM HC. In addition Cu^{2+} -treated HC (10 nM) was incubated with 0–80 nM Cu^{2+} -treated LC. Incubations were for 6 hours at 23°C in HEPES buffer after which factor VIII activity was measured by the factor Xa generation assay. Activity was determined as a function of added LC and data points were fitted to Eq.(1) by non-linear least squares regression. K_d was estimated and V_{max} was calculated by Eq. (2).

2.8. Statistical Analysis

Nonlinear least-squares regression analysis was performed by Kaleidagraph (Synergy, Reading, PA)

3. Results

3.1. HC and LC binding affinity is pH-dependent

Isolated HC and LC were purified from EDTA-treated factor VIII and employed to assess the inter-chain affinity over a range of pH values from 6.0–8.0. Binding was monitored by the reconstitution of functional factor VIII formed in the presence of Ca^{2+} based on the capacity of factor VIII to serve as a cofactor in the factor IXa-catalyzed generation of factor Xa. Samples of reconstituted factor VIII were diluted sufficiently so that buffer components and pH did not interfere with subsequent Xa generation reactions. Preparations of LC were typically of higher purity (>98%) as compared with the purified HC fraction (~95%), the latter of which contained trace levels of LC as visualized by SDS-PAGE (data not shown). For this reason, experimental protocols used fixed levels of HC that were titrated with LC (0–300 nM) in order to minimize activity derived from the LC present in the HC preparations. We performed experiments to examine the effects of incremental (0.25 pH units) increases between pH = 6 - 8 on factor VIII reconstitution. Figure 1 illustrates representative results obtained at pH 6.0 (A), 6.5 (B), 7.25 (C), and 8.0 (D). In all cases binding of HC could be saturated by LC. The affinity of this interaction was strongest at slightly acidic conditions with maximal values observed between pH 6.5 and 6.75 ($K_d = 3.8$ and 3.9 nM, respectively) (Figure 2A) and decreased at lower or higher pH values. While cofactor activity was significantly reduced at pH = 6.0, this parameter increased between pH = 6.25 and 8.0. The reconstituted activity observed at pH = 8 (~82 min^{-1}) was similar to values observed for an equivalent concentration of native factor VIII which ranged from 80–100 min^{-1} (data not shown). Thus the efficiency of factor VIII reconstituted from the isolated chains was at least 80% of the theoretical.

3.2. Inter-chain affinity is enhanced by Cu^{2+}

The presence of 0.5 μM (total) Cu^{2+} showed essentially no effect on the extent of activity regenerated, with the exception of an ~30% increase in the value observed at pH = 6.0 (Figure 2B). However, Cu^{2+} effectively increased the inter-chain affinity by ~2 to 5 fold over the range of pH values from 6.0 to 7.75. Interestingly, the effect of Cu^{2+} was maximal at physiological pH (= 7.25), with an observed reduction of K_d of approximately 5-fold. As pH increased from 7.25 to 8.0, this disparity in affinity values decreased and at pH 8.0 Cu^{2+} no significant effect on HC/LC affinity was observed. Control experiments showed that the concentration of Cu^{2+} employed did not effect subsequent thrombin activation or factor Xa generation reactions (data not shown).

3.3. Association kinetics of factor VIII chains

In order to examine the effects of Cu^{2+} on the kinetics of HC and LC interaction, association rates were measured by mixing HC (10 nM) and LC (200 nM). Since the concentration of LC was in excess, the rate of activity increase can be approximated to be proportional to the free HC concentration. Observed association rates were estimated from the fitted curve using a single exponential model. In the presence of Cu^{2+} the activity increased faster than that measured in its absence as reflected by an ~1.5 fold increase in observed association rate constant (Figure 3 and Table 1). Based on the K_d value at pH 7.25, association rate constants (k_{on}) were calculated using Eq.(4) in Methods. These results, presented in Table 1, show an ~1.6-fold greater k_{on} value obtained in the presence of Cu^{2+} . Due to the difficulty in controlling for free reactants, we were not able to measure directly values for the

dissociation rate constant (k_{off}). However, calculation of this parameter based upon k_{on} and K_{d} values yielded a k_{off} value for the HC/LC interaction that was ~3-fold greater in the absence of Cu^{2+} .

3.4. Affinity of Cu^{2+} for factor VIII

Since Cu^{2+} binding can be detected by monitoring regeneration of cofactor activity based upon enhanced dimer formation at low concentrations of HC and LC, we designed an experiment to estimate Cu^{2+} binding affinity for factor VIII. HC (10 nM) and LC (15 nM) were reconstituted in the presence of various concentrations of free Cu^{2+} at pH 7.25. To accurately assess the free Cu^{2+} level, an EGTA buffering system was employed as described in Materials and Methods to provide low levels of Cu^{2+} in equilibrium with high levels of chelated Cu^{2+} , the latter of which significantly exceeded the concentration of factor VIII chains in the assay. Based upon the inter-chain K_{d} at pH 7.25, inclusion of Cu^{2+} should yield an activity increase from ~27 to ~50 nM/min/nM HC, respectively. This ~2-fold disparity, observed at 15 nM LC, is illustrated in Figure 1C ($A_{\text{max}} = 23.4$ nM/min/nM HC). Thus, the differential activity in response to bound Cu^{2+} provides a sensitive, functional assay to assess its affinity. Results presented in Figure 4 show an initial activity value obtained in the absence of Cu^{2+} (31.9 nM/min/nM HC) that increased by an increment of ~15 nM/min/nM HC. This observed value was less than the calculated maximal increment ($A_{\text{max}} = 24.9$ nM/min/nM HC at ~10 fM Cu^{2+}) due to a contribution of the inhibitory component to this interaction that occurs at a higher concentration of Cu^{2+} (see below). From this data, we estimated a K_{d} for free Cu^{2+} binding of 5.3 ± 1.7 fM. Concentrations greater than ~50 fM free Cu^{2+} resulted in a decrease in cofactor activity to values below that observed for the absence of Cu^{2+} . This Cu^{2+} -dependent inhibition has been observed previously [16] and the mechanism for this effect is not known. Occupancy of the low affinity site(s) resulting in activity loss occurs with $K_{\text{d}} = 96.8 \pm 18.0$ fM. The incremental decrease in the activity ($B_{\text{max}} = 46.7$ nM/min/nM HC) due to the Cu^{2+} binding to the low affinity site represented ~80% of total activity ($A_{\text{max}} + C = 56.5$ nM/min/nM HC).

3.5. Effect of preincubation of isolated chains with Cu^{2+}

In order to examine whether the effect of Cu^{2+} to increase inter-chain affinity in factor VIII subunits resulted from Cu^{2+} binding a single chain or both chains simultaneously, HC and LC were pre-incubated individually with Cu^{2+} . Subsequently, factor VIII was reconstituted at pH 7.25 from the Cu^{2+} -treated chain plus the untreated complementary chain and activity was determined in a factor Xa generation assay. We did not detect a significant increase in HC/LC binding affinity using either Cu^{2+} -preincubated HC or LC (Figure 5A and 5B). Furthermore, preincubation of both chains with Cu^{2+} did not yield a significant increase in HC/LC binding affinity (Figure 5C). These results suggest that the high affinity Cu^{2+} site is contributed by residues on both HC and LC, and is generated following the association of these chains to form the factor VIII heterodimer.

4. Discussion

Results from this study demonstrate the pH-dependence of factor VIII HC and LC interaction and quantitate the binding affinity following reconstitution analysis over a range of pH values. Experiments were performed in the presence of Ca^{2+} , which is required for generation of the active cofactor conformation but makes no contribution to the inter-chain affinity [16]. Maximal affinity for the HC/LC interaction was observed at slightly acidic pH and was significantly decreased above pH = 7.25 or below pH = 6.25. This pH range approximates the pKa of His suggesting the inter-chain affinity may be closely related to the protonation state of the imidazol nitrogen of selected His residues. While the pKa of other amino acid residues may fall into this range based on their environment, several His residues

are postulated at the A1-A3 domain interface and we speculate potential contribution of these residues to the inter-chain affinity. The factor VIII structure model, based upon homology with ceruloplasmin, proposes the presence of several His residues including H94, H99, H1716, H1954, H1957, H1961, H2005, and H2007, at the A1-A3 domain interface [19]. Examination of the Hemophilia A database reveals point mutations at H94, H1954 and H1961 yield a hemophilia phenotype. Furthermore, a recent study employing a recombinant His1957→Ala point mutant indicated a 30% reduction in the inter-chain stability relative to wild type following thermal denaturation analysis [23]. Taken together, these data support a role for the charge status of some of these His residues in affecting interactions between A1 and A3 domains.

We speculate that the local environment including polar and hydrophobic interactions surrounding relevant (His) residues contributes to the observed pKa variations. The biphasic pattern of the inter-chain affinity as a function of pH, illustrated in Figure 2A, suggests involvement of multiple residues having different pKa values. One model consistent with these data suggests a residue(s) exhibiting relatively low pKa such that protonation is unfavorable for inter-chain affinity, whereas other residue(s) possess higher relative pKa with this protonation favorable for the affinity. Thus as pH increases, the former residue(s) are deprotonated while those of latter group remain protonated, leading to increased affinity. Maximal affinity is achieved with complete deprotonation of this group which occurs at pH ~6.5. Subsequent increases in pH reflect the deprotonation of the higher pKa residue(s) with concomitant reduction in affinity.

On the other hand the increase in reconstituted cofactor activity as judged by the $V_{\max}$ parameter showed a single-phase response. A steep activity increase was observed between pH 6.0 and 6.25 and this paralleled a similarly steep increase in inter-chain affinity, suggesting a coordination of these effects with the pKa value for the responsible residue(s) falling within this range. Activity increased modestly (~35%) between pH 6.25 and 8.0, suggesting that this effect was independent of interactions responsible for inter-chain affinity. This contention was further supported by the similarity in activity values obtained in the absence and presence of Cu^{2+} . These results are in conflict with past observations suggesting that Cu^{2+} made modest contributions to the specific activity of factor VIII [13,16]. We suggest these earlier observations in fact resulted from Cu^{2+} -dependent enhancement of the inter-chain affinity to yield a greater fraction of reconstituted factor VIII heterodimers rather than a direct effect of Cu^{2+} on specific activity. Another observation supporting the reliability of the current work is the high (~80%) recovery of factor VIII activity following reconstitution analysis. This high level of activity likely reflects minor improvements in our methods for chain purification.

We observed Cu^{2+} increases the HC/LC binding affinity as judged by a functional assay and this result was consistent with our prior studies assessing effects of the metal ion by fluorescence energy transfer [16]. That study estimated an ~100-fold increase in the interchain affinity observed in the presence of Cu^{2+} . However, the magnitude of this effect was likely an over-estimate based in part on large standard error values in the donor quenching data obtained at low chain concentration which define the upper limit for affinity. The present study demonstrated the Cu^{2+} -dependent affinity increase occurred over a wide range of pH tested (6.0 to 7.75). The reason for the disappearance of the response to Cu^{2+} at pH 8.0 is not clear. Since Cu^{2+} tends to form hydroxide precipitates at alkaline pH, this observation may reflect a reduced solubility of the metal ion. Maximal affinity enhancement (~5-fold) was observed at physiological pH (pH = 7.25) and appeared to result from modulation of both the association and dissociation rate constants. This affinity enhancement is similar to that observed for the A1/A3-C1-C2 dimer at pH 7.2 using fluorescence energy transfer (K_d ~50 and ~12 nM in the absence and presence of Cu^{2+} ,

respectively [23]), consistent with a minimal role for the A2 domain in the HC/LC interaction.

The coordination site(s) for copper ion(s) in factor VIII is not known and its location is controversial. Atomic absorption spectroscopy identified a single bound copper ion in factor VIII [20], and this ion was not associated with the isolated HC and LC following subunit dissociation. Thus it was predicted that the bound ion was located in a site between the A1 and A3 domains. Tagliavacca et al [12] detected Cu^{2+} in factor VIII by electron paramagnetic resonance only when factor VIII was treated by EDTA, suggesting that the copper ion present in factor VIII was diamagnetic Cu^+ , which was removed by EDTA and oxidized to detectable Cu^{2+} . Mutagenesis studies of the predicted type I copper binding site in the A1 domain showed marked reduction of activity and loss of HC/LC binding for a Cys310→Ser factor VIII. These authors concluded that the type I site in A1 domain coordinates Cu^+ in native factor VIII, and this coordination facilitates HC/LC association. Type I coordination of copper ion is characterized by a strong blue color due to charge transfer between the sulfide in cysteine and the copper ion, which is observed as intense absorption at 600 nm [28]. However, a high concentration factor VIII (~42 μM) neither appeared blue nor showed strong absorption at 600 nm (Wakabayashi and Fay, unpublished observations).

In this study we observed that the pre-incubation of separated chains with Cu^{2+} did not lead to increases in inter-chain affinity. Since the Cu^{2+} -binding affinity for the factor VIII heterodimer appears high ($K_d = 5.3 \text{ fM}$), this observation suggests that the functional, high affinity Cu^{2+} binding site is formed when both subunits are mixed. This contention is supported by our previous results in monitoring inter-chain affinity by fluorescence energy transfer [16]. Those experiments employed subunits modified by fluorophores at the free Cys residues (Cys310 and Cys2000, [29]) that contribute to the type I Cu^{2+} sites in the A1 and A3 domains, respectively. The fact that we observed Cu^{2+} -dependent, high affinity interaction with these reagents argues against the coordination of Cu^{2+} at the type I sites in affecting inter-chain affinity.

Previously we reported that the activity of reconstituted factor VIII increased following Cu^{2+} binding to a high affinity site ($K_d \sim 10\text{--}100 \text{ nM}$) and activity subsequently decreased by Cu^{2+} binding at a low affinity site ($K_d \sim 10\text{--}100 \mu\text{M}$) [16]. When we measured interactions of Cu^{2+} at pH 7.25, these stimulatory and inhibitory effects were observed at much lower Cu^{2+} concentrations. Results obtained in the previous study employed reaction mixtures containing ~1.5 μM BSA, which is necessary to prevent non-specific adsorption of factor VIII subunits. BSA is known to bind Cu^{2+} [30]. Therefore, the actual free Cu^{2+} concentrations were likely substantially lower than the values reported. In the present study, we use EGTA to control the level of free Cu^{2+} at physiological pH (= 7.25). High concentrations of Ca^{2+} (25 mM) were used in all reactions for factor VIII activity considerations [16]. However, the large difference in EGTA binding affinity for the metal ions (K_d values of 159 nM and 22.3 fM for Ca^{2+} and Cu^{2+} , respectively) did not affect its availability for chelating Cu^{2+} . Affinity values for Cu^{2+} -coordination in proteins typically occur within the fM range [30–32], and the estimated K_d for high and low affinity Cu^{2+} -binding sites in HC/LC (5.3 fM and 96.0 fM, respectively) fall within this range.

The K_d for HC/LC binding at physiological pH in the absence of Cu^{2+} was ~20 nM. Thus at plasma factor VIII concentration (~1 nM) the integrity of the heterodimer would not be maintained. We observed Cu^{2+} to reduce the K_d ~5-fold to ~3.8 nM. Although care was taken to preserve activity of individual subunits after separation, the conformational stability of separated subunits might not be equivalent as found in intact factor VIII, reflecting the slight reduction of affinity we measured. However, it is likely that copper ion coordination

in combination with binding of von Willebrand factor, which also increases the rate of reconstitution of HC and LC and stabilizes the heterodimeric structure [10,33], likely work in concert to promote the stability of the factor VIII heterodimer.

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The abbreviations used are

HC	factor VIII heavy chain
LC	factor VIII light chain
EDTA	ethylenediamine tetraacetic acid
MES	2-[N-morpholino]ethanesulfonic acid
MOPS	3-[N-morpholino]propanesulfonic acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
BSA	bovine serum albumin
PS	phosphatidylserine
PC	phosphatidylcholine
PE	phosphatidylethanolamine

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Figure 1.

Titration of factor VIII chains in the absence (open circles) or presence (closed circles) of Cu^{2+} .

HC (10 nM) was reacted with 0-300 nM LC at pH 6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, and 8.0 for 6 hours at 23°C in 20 mM MES (pH 6.0-6.5), MOPS (pH 6.75), or HEPES (pH 7.0-8.0) plus 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, 25 mM CaCl_2 , pH 7.25. The figure shows results obtained at pH 6.0 (A), 6.5 (B), 7.25 (C), and 8.0 (D). Reconstituted activity was determined by factor Xa generation assay and lines were drawn from the curve fit as described in Materials and Methods. Error bars are drawn from the values of the standard errors of three separate determinations.

Figure 2.

A) Dissociation constant (K_d) and B) Activity at chain saturation (V_{max}) at various pH values.

The K_d and V_{max} for the factor VIII chain association as shown in Figure 1 were estimated by nonlinear least squares regression as described in Materials and Methods and plotted as a function of pH. Error bars are drawn from the values of asymptotic errors of nonlinear least squares regression.

Figure 3.

Association kinetics of factor VIII HC and LC. Factor VIII chain association in the absence or presence of Cu^{2+} .

HC (10 nM) or LC (200 nM) was separately incubated for 4 hours at 23°C in 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, 25 mM CaCl_2 , pH 7.25 in the absence or presence of 0.5 μM Cu^{2+} . Reactions were initiated by mixing LC and HC solutions. Aliquots were taken at indicated times and factor VIII activity was measured using the factor Xa generation assay. Curves were drawn from the fitted data according to single exponential as described in Materials and Methods. Error bars are drawn from the values for the standard errors of three separate determinations.

Figure 4.

Titration of factor VIII reconstitution by Cu²⁺.

HC (10 nM) and LC (15 nM) in 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, 25 mM CaCl₂, pH 7.25 the presence of 0-2.8 pM free Cu²⁺ was incubated for 6 hrs at 23°C. The indicated free Cu²⁺ concentrations were calculated as described in Materials and Methods. Reconstituted activity was determined by factor Xa generation assay and lines were drawn from the curve fit as described in Materials and Methods. Error bars are drawn from the values for the standard errors of three separate determinations.

Figure 5.

Effect of pre-incubation of HC or LC with Cu²⁺.

Cu²⁺ (1 μ M) was added to 1 μ M HC or 1 μ M LC in 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, pH 7.25 for 1 hour, followed by dialysis into 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, pH 7.25. A) 10 nM HC or Cu²⁺-treated HC was reacted with 0–80 nM LC. B) 10 nM LC or Cu²⁺-treated LC was reacted with 0–80 nM HC. C) 10 nM Cu²⁺-treated HC was reacted with 0–80 nM Cu²⁺-treated HC. Reactions were run for 6 hours at 23°C in 20 mM HEPES, 0.3 M KCl, 0.01% Tween 20, 0.01% BSA, 25 mM CaCl₂, pH 7.25. Reconstituted activity was determined by factor Xa generation and lines were drawn from the curve fit as described in Materials and Methods. Error bars are drawn from the values for

the standard errors of three separate determinations. Values for $V_{\max}$ (nM/min/nM HC) and K_d (nM) respectively were 74.7 ± 4.2 and 16.4 ± 2.7 (HC/LC), 74.6 ± 2.8 and 16.4 ± 1.8 (Cu^{2+} -HC/LC), 69.8 ± 2.6 and 15.2 ± 1.6 (LC/HC), 72.4 ± 1.8 and 17.7 ± 1.2 (Cu^{2+} -LC/HC), and 75.1 ± 3.0 and 14.7 ± 1.8 (Cu^{2+} -HC/ Cu^{2+} -LC).

Table 1HC/LC binding parameters in the presence or absence of Cu^{2+} at pH 7.25

	Cu^{2+} (-)	Cu^{2+} (+)
observed association rate constant (k_{ob}) $\times 10^2$ (min^{-1})	6.56 ± 0.29	9.93 ± 0.33
association rate constant (k_{on}) $\times 10^{-5}$ ($\text{M}^{-1}\text{min}^{-1}$)	2.99	4.87
K_{d} (nM)	19.6 ± 1.5	3.8 ± 0.5
calculated dissociation rate constant (k_{off}) $\times 10^3$ (min^{-1})	5.86	1.85

k_{ob} was estimated by non-linear least squares regression on the data shown in Figure 3 using the formula shown in Materials and Methods. k_{on} and k_{off} were further calculated based on k_{ob} , K_{d} , and concentration of excess ligand (LC) as described in Materials and Methods.