Effects of Copper on the Structure and Function of Factor VIII Subunits: Evidence for an Auxiliary Role for Copper Ions in Cofactor Activity†

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ABSTRACT: Factor VIII, a divalent metal ion-dependent heterodimer, contains a single copper atom, but the role of this metal in the structure and function of the cofactor is unclear. Earlier results showed that the dissociated heavy and light chains of factor VIII could be recombined in the presence of Ca(II) or Mn(II) but not Cu(II) to yield functional protein [Fay, P. J. (1988) *Arch. Biochem. Biophys. 262*, 525−531]. Inclusion of Cu(I) or Cu(II) inhibited the Mn(II)- or Ca(II)-dependent reconstitution of factor VIII with an IC_{50} ∼10 μM. The heavy chain was the susceptible subunit with inhibition by copper ion resulting from its reduced affinity for light chain. On the other hand, Mn(II)-dependent factor VIII reconstitutions performed with Cu(II) light chain and native heavy chain occurred at an accelerated rate (∼10-fold) and yielded an enhanced activity (∼50%), likely reflecting an increased specific activity of the heterodimer. Cu ions enhanced the activity of EDTA-treated factor VIII in the presence of Ca(II) but not in its absence, suggesting that EDTA-treated factor VIII is not equivalent to separated subunits and that copper ions are auxiliary to ions that mediate reconstitution. Conformational analyses showed that the ellipticities and extrinsic fluorescence of both subunits were differentially affected by Cu(II) and Mn(II). These structural effects were fully reversed by EDTA. The metal ions had little if any effect on the conformation of intact factor VIII or the A1/A3-C1-C2 dimer. Mn(II) and Cu(II) stabilized the factor VIII light chain, and the latter stabilized the A1 subunit derived from the heavy chain, yielding similar thermal denaturation profiles that were distinct from that observed for the Ca(II)-stabilized subunits. Thus both subunits of factor VIII bind copper ions, and the effects of this binding differ from the interactions observed with Ca(II) or Mn(II). These data support a model where copper in factor VIII likely functions to increase specific activity of the heterodimer rather than directly mediating the intersubunit interaction.

Factor VIII, the blood coagulation protein deficient or defective in individuals with hemophilia A, is synthesized as a 300-kDa precursor protein (*1*, *2*) with domain structure A1-A2-B-A3-C1-C2 (*3*). It is processed to a series of divalent metal ion-dependent heterodimers (*4*−*6*), produced by cleavage at the B-A3 junction generating a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains). Cleavage at additional sites within the B domain results in variable sized heavy chains minimally represented by the contiguous A1-A2 domains. The two chains can be separated by chelating reagents (*4*, *6*) and isolated following ion exchange chromatography. The separated chains have no activity, but factor VIII activity can be reconstituted by combining the subunits in the presence of divalent metal ions such as Ca(II) and Mn(II) (*7*, *8*).

Thrombin converts factor VIII to the active cofactor (factor VIIIa)[1] by limited proteolysis (*9*). Thrombin cleaves factor VIII heavy chain at Arg^{740}, which liberates the B domain fragments, and at Arg^{372}, which bisects the contiguous A1-A2 domains into the A1 and the A2 subunits.[2] Cleavage of the light chain 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-C1-C2 (*10*, *11*). The A1 and A3-C1-C2 subunits retain the divalent metal ion-dependent linkage and can be isolated as a stable A1/A3-C1-C2 dimer. The A2 subunit is weakly associated with the dimer through electrostatic interactions (*11*, *12*).

The A domains of factor VIII share sequence homology with the A domains of factor V and the copper-binding protein, ceruloplasmin. Together, these proteins constitute a family of homologous proteins (*13*). Ceruloplasmin

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[1] Abbreviations: BSA, bovine serum albumin; bis-ANS, bis-anilinonaphthalsulfonic acid; CD, circular dichroism; factor VIIIa, activated factor VIII; and Mes, 2-(*N*-morpholino)ethanesulfonic acid.

[2] The factor VIIIa nomenclature is based on the domainal structure designated by A1-A2-B-A3-C1-C2. Factor VIIIa subunits are designated as A1 (residues 1−372), A2 (residues 373−740), and A3-C1−C2 (residues 1690−2332) with noncovalent subunit associations denoted by a slant (/) and covalent associations by a hyphen (-).

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contains six copper ions liganded in multiple configurations (see ref *14* for review). Atomic absorption spectroscopy indicates 1 mol of copper/1 mol of factor V (*15*) or factor VIII (*16*). Spectral properties of these proteins suggest that the bound copper is not in a type I or type III site. This ion is lost following dissociation of the A1 and A3-C1-C2 subunits of factor VIIIa (*16*).

Recent homology modeling of the factor VIII A domains using nitrite reductase (*17*) and ceruloplasmin (*18*) predict that the copper in factor VIII is bound in a type II configuration at the interface of A1 and A3 subunits. Thus, copper has been proposed to function in the association of heavy and light chains. Recently, Cu(I) has been identified as the ion form in factor VIII (*19*). That study also suggested that Cu(I), and to a lesser extent Cu(II), reconstituted factor VIII activity from the EDTA-treated material. However, earlier results from our laboratory (*7*) failed to show reconstitution of factor VIII activity from isolated subunits in the presence of Cu(II). That study was limited to assessment of activity following recombination of the subunits. The effect of metal ion on structure was not considered at that time because of limitations in reagents.

In the present study, we attempt to identify the functional role of copper in factor VIII activity and compare its effect on subunit structure to other divalent metal ions that promote subunit association. These results show that both factor VIII subunits can bind copper and that this binding results in significant conformational changes that are disparate to those produced by Mn(II) and Ca(II). Furthermore, we show by reconstitution analyses that copper ion alone is unable to reconstitute active factor VIII. However, under appropriate conditions, copper ions enhance the specific activity of the factor VIII heterodimer.

MATERIALS AND METHODS

Reagents. Recombinant factor VIII was generously provided as Kogenate by Dr. Jim Brown of Bayer Corporation. Human serum albumin was removed from factor VIII following modification of a previously described procedure (*20*). Briefly, factor VIII powder was reconstituted with 20 mM Mes (pH 6.0), 0.1 M NaCl, 1 mM $CaCl_2$, and 0.01% Tween-20 (10 mL/600 unit of factor VIII). The material was applied to a column of S-Sepharose (1 mL/1000 unit of factor VIII) equilibrated in the above buffer. The column was washed with the equilibration buffer (10−20 column volumes) until the A_{280} of eluant was <0.01 and factor VIII was batch eluted using the above buffer containing 1 M NaCl. Yields were typically >90%, and the material was virtually free of albumin. Spectral examination of factor VIII (4 μM) showed no absorption peaks at 610 and 330 nm, suggesting the absence of type I and type III copper in the protein. Factor VIII subunits were prepared from recombinant factor VIII as previously described (*21*). Following dissociation of the subunits, all subsequent chromatography buffers were supplemented with 0.1 mM EDTA, and dialysis buffers contained (insoluble) Chelex-100. Human α-thrombin was obtained from Enzyme Research Labs. The dipotassium salt of bis-ANS was obtained from Molecular Probes and used without further purification. The stock solutions of bis-ANS were prepared in buffer (20 mM Tris and 0.1 M NaCl at pH 7.0) and filtered, and the concentration was determined by absorbance at 385 nm using an extinction coefficient, $\epsilon_{385} = 16790\ cm^{-1}\ M^{-1}$. Cupric chloride ($CuCl_2$) and cuprous chloride (CuCl) were obtained from Sigma, and Chelex-100 was obtained Bio-Rad.

Assays. Factor VIII activity was determined using a one-stage clotting assay with substrate plasma that had been chemically depleted of factor VIII activity (*22*). Factor VIII was reconstituted from isolated heavy and light chains (see legends for concentrations and reaction times) in a reaction containing 20 mM Tris (pH 7.2), 0.1 M NaCl, 0.01% Tween-20, and 5 mM $MnCl_2$. EDTA-treated factor VIII was prepared by reacting the protein (∼100 unit/mL in 1 mg/mL of BSA) with EDTA (50 mM) overnight at room temperature. Samples were subsequently dialyzed against buffer containing 20 mM Tris (pH 7.2), 0.1 M NaCl, and 0.01% Tween-20 in the absence or presence of 2.5 mM $CaCl_2$. Residual activity for the above samples were <0.5 and ∼8 unit/mL, respectively. Copper-treated factor VIII and subunits were prepared as indicated in the legends and used within 24 h of preparation. All reactions were run at room temperature. Other additions are as described in the figure legends. Protein concentrations were determined by the Coomassie blue dye binding method of Bradford (*23*) using bovine serum albumin as the standard.

CD Spectroscopy. CD spectra in the range of 200−250 nm at room temperature were recorded on a Jasco-720 spectropolarimeter. Samples were measured in cylindrical fused quartz cells with the path length of 0.5 mm, using 16 s response time, 5 nm/min scan speed, and 0.1 nm step size. The concentration of protein used for these studies was approximately 1−5 μM in 20 mM Tris (pH 7.0) and 0.1 M NaCl. Observed ellipticities were converted to mean residue ellipticity and are expressed in deg cm^{-2} $dmol^{-1}$.

Fluorescence Spectroscopy. Fluorescence measurements were performed using a SPEX Fluorolog 1681 spectrometer. The concentration of protein used for these studies was approximately 200 nM. Reaction conditions are as described in the appropriate figure legends. The excitation and emission slit widths were set to 2 and 6 mm, respectively. Samples with bis-ANS were excited at 385 nm, and emission spectra were recorded between 400 and 600 nm. The fluorescence intensities of the samples were corrected for the absorption of dye by the relation (*24*), $F_{corr} = F_{obs}$ antilog-$(OD_{ex} + OD_{em}/2)$, where OD_{ex} and OD_{em} are the optical densities at excitation and emission wavelengths, respectively. All spectra were corrected with respect to the spectra of buffer background and dilution effects.

RESULTS

Effect of Cu Ions on the Reconstitution of Factor VIII and Subunit Activity. Factor VIII subunits were prepared following combination Mono S/Mono Q chromatography. All chromatography buffers were supplemented with 0.1 mM EDTA, and dialysis solutions contained Chelex-100 to adsorb any trace metals present. Since subunit dissociation results in loss of the bound copper (*16*), these steps were performed to prevent reassociation of the copper or any other trace metal ion. Resultant subunits were judged pure by SDS−polyacrylamide gel electrophoresis (data not shown). Our earlier experiments showed that Cu(II) alone was ineffective in supporting the regain of factor VIII activity following mixing

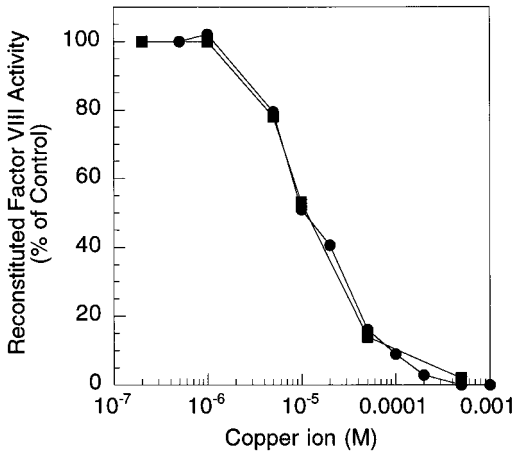

FIGURE 1: Effect of copper ions on the reconstitution of factor VIII activity. Factor VIII reconstitution reactions were performed as described in Materials and Methods and contained heavy chain and light chain (1 μM each) plus the indicated levels of Cu(I) (squares) or Cu(II) (circles). Reactions were initiated with 5 mM $MnCl_2$ and were run for 90−150 min after which no further increase in factor VIII activity was observed. Data points represent the average of at least two separate determinations. Standard deviations were typically less than 15% of the mean.

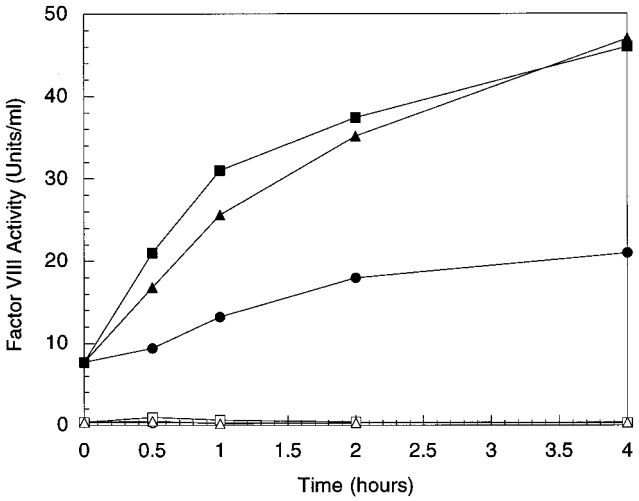

FIGURE 2: Effect of copper ions on EDTA-treated factor VIII in the absence and presence of $CaCl_2$. Factor VIII (~100 unit/mL) in buffer containing BSA (1 mg/mL) was reacted with EDTA (50 mM) as described in Materials and Methods. Following this treatment, samples were dialyzed in buffer containing 2.5 mM $CaCl_2$ (closed symbols) or in its absence (open symbols). Initial factor VIII levels were ~8 and <0.5 unit/mL, respectively. Reactions contained no additions (circles), 10 μM CuCl (squares), or 10 μM $CuCl_2$ (triangles). Data points represent the average of at least two separate determinations. Standard deviations were typically less than 15% of the mean.

of heavy and light chains (*7*). Similar experiments using Cu(I) were unsuccessful in regenerating active factor VIII (data not shown). Thus, the function of this metal in factor VIII structure is unclear. To determine whether the copper ion served an auxiliary role in subunit association, reconstitution assays were performed in the presence of 1 μM subunits plus variable levels of either CuCl or $CuCl_2$ (Figure 1). Reactions were initiated by addition of a divalent metal ion known to promote subunit reconstitution [Mn(II), Figure 1; Ca(II), data not shown]. In the absence of copper (data not shown), reconstitution of activity yielded approximately 1000 unit/mg of factor VIII protein. This value was consistent with our earlier observations (*7*, *21*) and severalfold less than the specific activity of purified factor VIII (~5000 unit/mg; *25*), suggesting incomplete reconstitution and/or that the reconstituted material was less active than the native protein. The presence of Cu(I) or Cu(II) at concentrations below that of the factor VIII subunits had no effect on the reconstitution of activity (Figure 1). However, higher levels of copper showed marked inhibition of the reaction with 50% inhibition observed at ~10 μM copper ion. The reconstitution of factor VIII activity was virtually eliminated at >0.1 mM copper ion. Similar results were obtained when Mn(II) was replaced by Ca(II) (data not shown), indicating that the copper-dependent inhibition was independent of the divalent metal ion mediating factor VIII reconstitution. These results suggest a high affinity (μM) interaction between copper ions and factor VIII subunit(s) that markedly inhibits the regeneration of active factor VIII.

Recently, Tagliavacca et al. (*19*) showed that 10 μM Cu(I) or Cu(II) increased the activity of EDTA-treated factor VIII by ~4- or ~2-fold, respectively. However, dialysis of the EDTA-treated material prior to copper addition used a buffer containing 2.5 mM $CaCl_2$, a divalent metal ion that alone is capable of supporting the reconstitution of factor VIII (*7*). The initial factor VIII activity of that sample was consistent with a specific activity of ~1000 unit/mg of factor VIII protein, a value similar to that observed for factor VIII reconstitution from isolated subunits. To reconcile these data relative to the above experiments using isolated subunits, a similar experiment was performed using EDTA-treated factor VIII that had been dialyzed in the presence of 2.5 mM $CaCl_2$ as well as in its absence.

Consistent with the data of Tagliavacca et al. (*19*), our results show that both Cu(I) and Cu(II) stimulate the activity of EDTA-treated factor VIII that had been "partially reconstituted" following dialysis using a Ca(II)-containing buffer (Figure 2, closed symbols). In our hands, Cu(I) and Cu(II) were essentially equivalent, yielding a ~4-fold increase in the regain of cofactor activity. However, neither ion was capable of regenerating significant factor VIII activity when Ca(II) had been omitted from the dialysis step (Figure 2, open symbols). These results suggest that while copper ions alone are unable to facilitate the regeneration of activity from metal ion-depleted material, copper does enhance the specific activity of the preformed, Cu-deficient factor VIII heterodimer. Given the similarity of results using Cu(I) and Cu(II) and the tendency of Cu(I) to oxidize, subsequent experiments unless indicated used Cu(II).

The results shown in Figure 1 indicated at least one factor VIII subunit was susceptible to inhibition by low concentrations of copper ions. To identify the subunit, the following experiment was performed. Factor VIII light and heavy chains were separately reacted with Cu(II) (100 μM) for 1 h at room temperature. The subunits were then dialyzed to remove the free and/or weakly bound Cu(II). Reconstitution reactions were performed using the native and Cu(II)-treated subunits in the presence of $MnCl_2$. Results of this study, shown in Table 1, indicated that reaction of Cu(II) with heavy chain reduced its capacity to yield functional factor VIII following reaction with light chain by ~90%. However, inclusion of equal concentrations of Cu(II)-treated and native heavy chains (1 μM each) resulted in the generation of

Table 1: Effect of Cu(II) Treatment of Individual Factor VIII Subunits on Reconstitution

condition[a]	activity (units/mL)[b]
0.5 μM LC[c]	
+ 1 μM HC	204
+ 1 μM Cu-HC	19
+ 1 μM Cu-HC + 1 μM HC	232
0.5 μM HC	
+ 1 μM LC	144
+1 μM Cu-LC	197
1 μM Cu-LC + 1 μM Cu-HC	23

[a] Reactions were as described in Materials and Methods in buffer containing 50 mM Tris, pH 7.2, 0.1 M NaCl, and 0.02% Tween-20, initiated with 5 mM $MnCl_2$ and run for 2−3 h. [b] Average of at least two determinations. Standard deviations were less than 15% of the mean. [c] LC, light chain; HC, heavy chain; Cu-, Cu(II)-treated subunit.

activity similar to that in the control reaction. This result suggested that the Cu(II) heavy chain possessed a markedly reduced affinity for light chain as compared with the native heavy chain, rather than the alternative explanation that Cu(II) heavy chain associated with light chain to yield an inactive heterodimer. Conversely, Cu(II) light chain consistently yielded greater activity in reconstitution assays as compared with native light chain. Since heavy chain was limiting in these reconstitution reactions, the enhancement likely reflected an increase in cofactor specific activity rather than an increase in the number of factor VIII molecules formed. The reasons for the disparate functional effects of Cu(II) on the factor VIII heavy and light chains are not known but may correlate to Cu(II)-induced alterations in their conformations (see below). Comparison of the two control reactions show that, for a given concentration, limiting light chain yielded more activity (204 unit/mL) than limiting heavy chain (144 unit/mL). We have observed this disparity in previous studies (*7*, *21*). The reason for this is not clear but may reflect a lability in the heavy chain resulting in a population of inactive molecules.

A time course of factor VIII reconstitution was performed to determine whether Cu(II) treatment of light chain also affected its rate of interaction with heavy chain. Reconstitution assays containing heavy chain and an equimolar concentration of native or Cu(II) light chain were sampled over an early time course, and the amounts of regain in factor VIII activity were determined. Results from this experiment, shown in Figure 3, indicated that the rate of reconstitution was accelerated in the reaction employing the Cu(II) light chain. Comparison of initial reconstitution rates using the linear portions of the two curves suggested that the rate of reformation of factor VIII was approximately 12-fold faster using the Cu(II) light chain.

Metal Ion Effects on Factor VIII Activity. Since the factor VIII subunits showed a marked response to the presence of copper ions, we performed an experiment to determine whether Cu(II) affected the activity of native factor VIII. Figure 4 shows results from the reaction of 0.2 μM factor VIII (dialyzed in Tris buffer plus Chelex) in the presence of increasing levels of Cu(II), Mn(II), or Ca(II). The latter two ions resulted in a slight stimulation and/or stabilization of activity, whereas Cu(II) was inhibitory. The concentration of Cu(II) resulting in half-maximal inhibition of factor VIII activity (∼2 mM) was significantly greater than the concentration required to yield 50% inhibition of the isolated factor VIII heavy chain (∼10 μM) in a reconstitution assay. This result suggested that the Cu(II)-dependent inhibition of the intact molecule occurred by a different mechanism than the loss of subunit function.

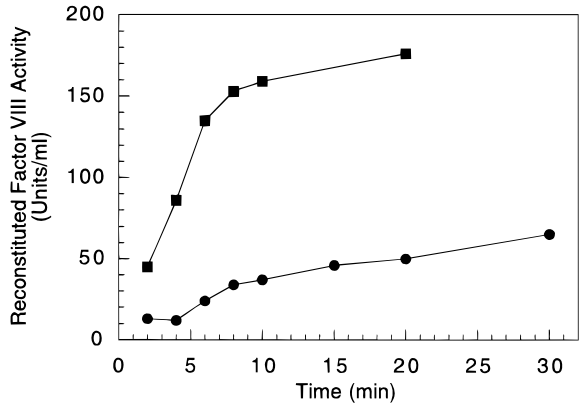

FIGURE 3: Effects of Cu(II) treatment of light chain on the rates of factor VIII reconstitution. Reactions contained 1 μM heavy chain and either 1 μM light chain (circles) or 1 μM Cu(II)-treated light chain (squares) that had been dialyzed to remove unbound Cu(II). Reconstitutions were run as described in Materials and Methods, and activity was determined at the indicated times using a one-stage clotting assay.

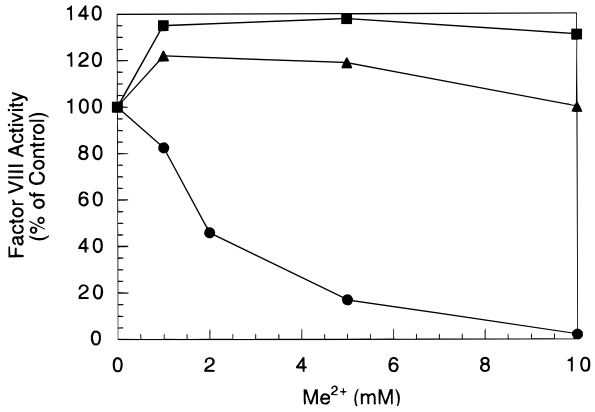

FIGURE 4: Effect of divalent metal ions of factor VIII activity. Factor VIII (200 nM) was reacted with the indicated concentrations of Ca(II) (triangles), Mn(II) (squares), or Cu(II) (circles) for 30 min at room temperature. Activity was assayed in a one-stage clotting assay.

Effect of Divalent Metal Ions on the Secondary Structure of Factor VIII Subunits. The above results show marked and disparate alterations in subunit function following reaction with copper ions. In an attempt to correlate these changes with conformation, we examined the CD spectra of the Cu(I)-, Cu(II)-, and Mn(II)-treated and native subunits. For these and subsequent analyses, subunits were treated with the indicated metal ion overnight [0.1 mM Cu ions; 10 mM Mn(II)] followed by dialysis against a Tris buffer containing Chelex resin to adsorb unbound metal ions. Samples were used within 24 h of preparation. This method was employed so that metal ion was retained only at the high-affinity sites in the proteins. CD revealed that Cu(I) and Cu(II) light and heavy chains possess significantly reduced ellipticities as compared with the native subunits (Figure 5), suggesting that each subunit was capable of binding the copper ions and that this binding affected the secondary structure of the subunits. These effects appeared global in that the reductions

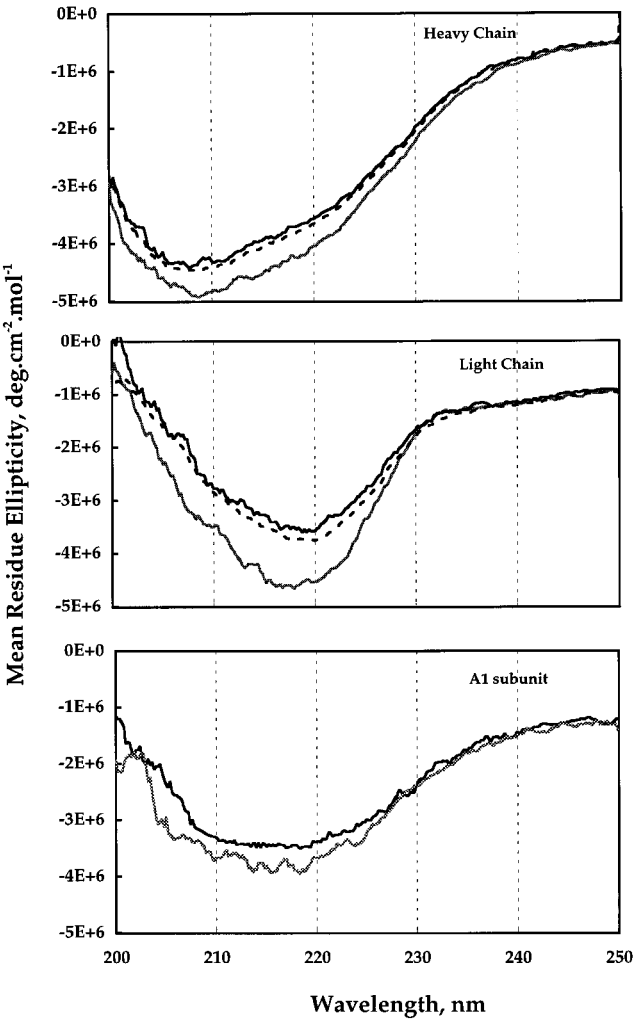

FIGURE 5: Effects of Cu(I) and Cu(II) on secondary structure of factor VIII subunits. CD spectra of heavy chain, light chain, and A1 subunit in the absence (gray line) and presence of bound Cu(II) (black line) and bound Cu(I) (dashed line). Spectra were obtained as described in Materials and Methods.

occur over a wavelength range from >220 to ~200 nm. Removal of copper ions with EDTA (5 mM) resulted in the recovery of the original ellipticities (results not shown), indicating that the copper-induced conformational changes in each subunit were reversible. The copper ion-dependent conformation change observed for the heavy chain was likely restricted to within the A1 domain based upon the observation that the Cu(II)-A1 subunit showed a similar response as heavy chain by CD (Figure 5), whereas the CD pattern observed for the isolated A2 subunit was unchanged by Cu(II) treatment (data not shown). Furthermore, treatment of intact factor VIII or the A1/A3-C1-C2 dimer with Cu(II) as described above did not affect their conformations (data not shown), suggesting the absence of high-affinity site(s) that modulate conformation in the intact dimer forms of the molecule. These observations further suggested that the copper-dependent loss of factor VIII activity observed in Figure 4 did not result from alterations in gross conformation or dissociation of the factor VIII subunits but rather reflected another mechanism, possibly resulting from occupancy of weak affinity sites.

The effects of Mn(II) on the CD of factor VIII light and heavy chains were similar to those observed with Cu(II) but

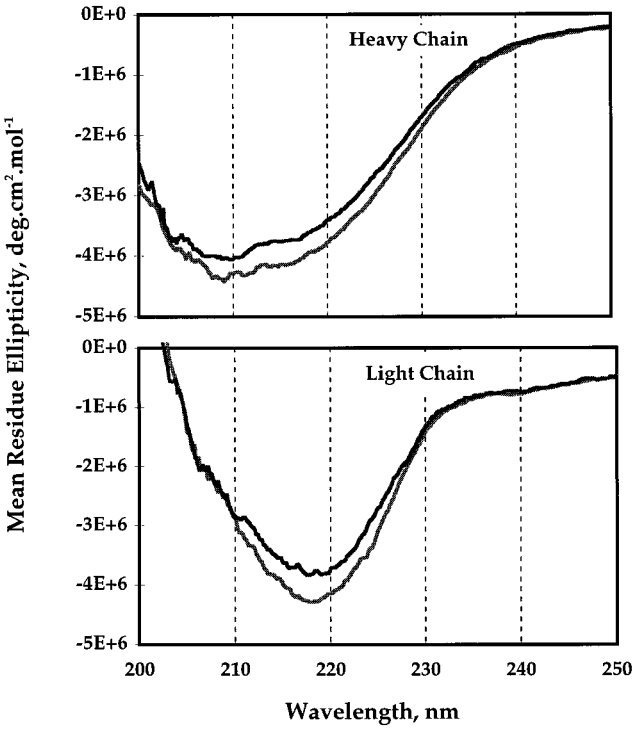

FIGURE 6: Effects of Mn(II) on secondary structure of factor VIII subunits. CD spectra of heavy chain and light chain were performed in the absence (gray line) and presence (black line) of bound Mn(II). Spectra were obtained as described in Materials and Methods.

not equivalent (Figure 6). Compared with the CD spectra obtained for the Cu-treated light chain, Mn(II)-treated light chain exhibited a lesser reduction in ellipticity that was restricted to a wavelength of 210−220 nm. Below 210 nm, the spectra of Mn(II)-treated and untreated light chains were indistinguishable. Results with heavy chain also showed a reduced change in ellipticity over a more restricted wavelength range as compared with that observed for Cu(II). Similar to results obtained with Cu(II), the A1/A3-C1-C2 dimer and factor VIII did not show any CD spectral changes on addition of Mn(II) (results not shown), indicating that reaction of the dimers with these ions did not affect their gross conformation.

Extrinsic Fluorescence Properties following Divalent Metal Ion Binding. The fluorescence emission of the apolar probe, bis-ANS, is of low intensity with a maximum at 533 nm in aqueous medium (*26*). When bis-ANS binds to a hydrophobic site, its fluorescence intensity increases severalfold and emission maximum shifts toward the blue dependent upon the environment around the hydrophobic site (*27*). In a recent report (*28*), we identified exposed hydrophobic sites on the surface of factor VIII subunits with this reagent. We showed that the high affinity bis-ANS sites localized to the heavy and light chains were lost following subunit reassociation to form the heterodimer, suggesting a hydrophobic component to the intersubunit interaction. Thus, these apolar sites may be in close proximity to the putative divalent metal ion binding site(s) that contribute to the intersubunit interaction.

In the present study, we used bis-ANS bound to these hydrophobic sites as a probe for the metal ion interactions with the subunits of factor VIII (Figure 7). Addition of 1 mM Cu(II) resulted in significant reduction in the fluores-

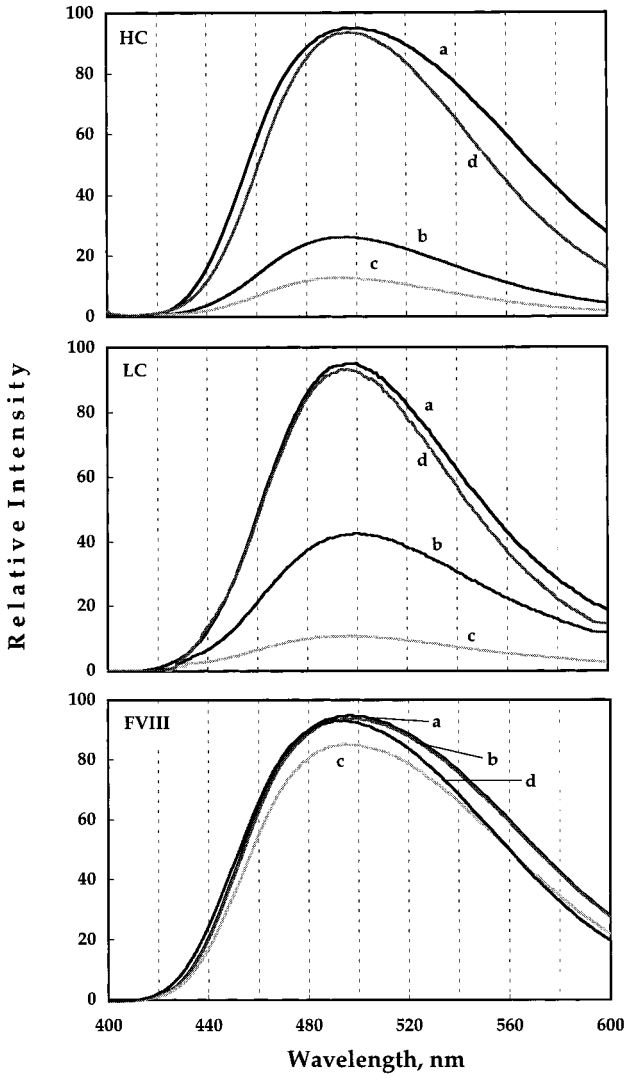

FIGURE 7: Fluorescence emission spectra of bis-ANS bound to factor VIII or isolated subunits. The concentration of protein was 0.2 μM in 0.02 M Tris, pH 7, and 0.1 M NaCl. Bis-ANS was at 10 μM. The excitation wavelength was 385 nm. Emission spectra were obtained for heavy chain (HC), light chain (LC), and factor VIII (FVIII) in the absence (a) or presence of 1 mM Cu(II) (b), 5 mM Cu(II) (c), and 1 mM Cu(II) plus 5 mM EDTA (d).

cence intensity for bis-ANS bound to light chain (50%) and heavy chain (70%). A higher concentration (5 mM) of Cu(II) virtually eliminated the fluorescence signal observed with both subunits. The magnitude of fluorescence reduction was significantly greater than the ~20% reduction we previously observed with excess Ca(II) (*28*). The presence of 20 mM Mn(II) resulted in a fluorescence effect of similar magnitude to that observed earlier with Ca(II) (results not shown). Inclusion of EDTA reversed the effects of Cu(II) (and Mn(II); results not shown) and restored the original level of bis-ANS fluorescence. These data suggested either that Cu(II) binds at hydrophobic sites replacing the bound bis-ANS, thereby resulting in a decreased fluorescence intensity, or that Cu(II) binding occurs close to the hydrophobic site and quenches the fluorescence of the dye. The effect of Cu(II) on the bis-ANS fluorescence properties of heterodimeric factor VIII differed from that of the isolated subunits. No effect on the fluorescence intensity of the dye was observed following the addition of 1 mM Cu(II), whereas 5 mM metal

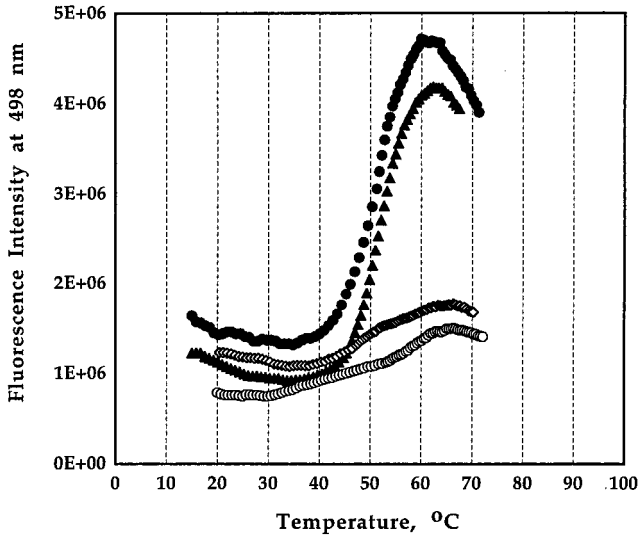

FIGURE 8: Thermal denaturation of factor VIII light chain. Samples contained 0.2 μM light chain, 10 μM bis-ANS in 0.02 M Tris (pH 7), and 0.1 M NaCl. Excitation and emission wavelengths were 385 and 500 nm, respectively. Emission was determined as a function of temperature for light chain in the absence of metal ion (closed circles), Ca(II) light chain (triangles), Mn(II) light chain (diamonds), and Cu(II) light chain (open circles).

ion resulted in a slight decrease in the fluorescence, which was reversed by EDTA. These results suggested a weaker affinity of Cu(II) for factor VIII as compared with Cu(II) affinity for the subunits and were consistent with the concentrations of Cu(II) required to affect the activities of isolated subunits as compared with the intact protein.

Effect of Metal Ions on Light Chain Stability. Thermal denaturation was monitored as a function of exposure of hydrophobic moieties in the presence of bis-ANS. Figure 8 shows temperature dependence of the bound bis-ANS fluorescence intensity in the light chain. Above 35 °C there is a large increase in the fluorescence intensity that decreases steadily beyond 60 °C. The increase in the fluorescence intensity of bound bis-ANS reflects exposure of more hydrophobic sites as a result of unfolding of the protein. The denaturation temperature was approximated as the temperature at which the fluorescence intensity reached a value that was midway between the minimum and maximum fluorescence intensity. A single thermal transition was observed for the factor VIII light chain with a T_m value of 50.5 °C. Factor VIII light chain was treated with the indicated metal ions [Cu(II), Mn(II), and Ca(II)] followed by dialysis against buffer plus Chelex to remove the unbound and weakly associated metal. A similar, sharp transition was observed for Ca(II) light chain that yielded an increased T_m of 53 °C. However, when light chain was treated with Cu(II), we observed a low-intensity biphasic thermal transition, with T_m values of 42 and 58 °C. Mn(II) light chain yielded a similar biphasic transition with T_m values of 47 and 59 °C. Changing the concentration of bis-ANS had no detectable affect on the melting curves (data not shown). The low intensity of bis-ANS fluorescence observed in the presence of Cu(II) and Mn(II) indicated that unfolding was limited to restricted regions in the protein. Assuming a standard error of ±2 °C for the biphasic transitions, the T_m values determined in the presence of Mn(II) and Cu(II) were essentially equivalent.

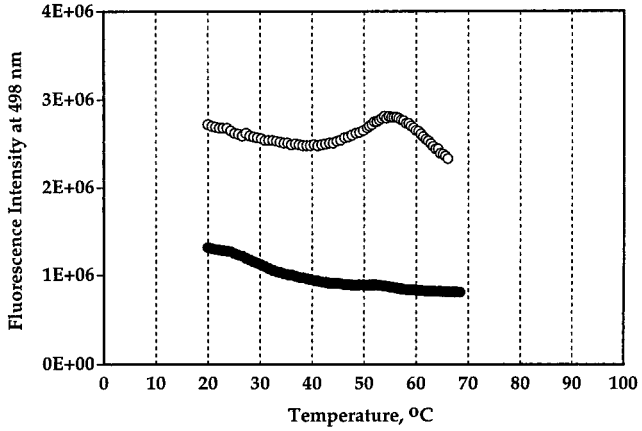

FIGURE 9: Thermal denaturation of A1 subunit. The concentration of the protein was 0.2 μM in 10 μM bis-ANS in 0.02 M Tris (pH 7) and 0.1 M NaCl. Excitation and emission wavelengths were 385 and 500 nm, respectively. Emission was determined as a function of temperature for A1 subunit in the absence (open circles) and presence of Cu(II) (closed circles).

Similar analysis of the native heavy chain and A2 subunit failed to yield an increase in bis-ANS binding as a function of temperature (results not shown). The reason for this is not clear but may be due to aggregation of the subunits. To eliminate potential aggregation problems related to the A2 domain of heavy chain, we utilized A1 subunit for thermal denaturation analysis (Figure 9). In the absence of added metal ion, a low-intensity thermal transition (T_m = 48 °C) was observed, indicating that unfolding was limited to a restricted region in this subunit. The presence of Cu(II) essentially eliminated the increase in fluorescence intensity. Limitations in amounts of A1 subunit precluded a more thorough examination of metal ions on its thermal stability. Taken together, these results suggest that all three metal ions increase the stability of the light chain and Cu(II) increases stability of A1 subunit toward thermal denaturation. However, the sites occupied by Ca(II) appear different from the sites occupied by Mn(II) and Cu(II).

DISCUSSION

A divalent metal ion is essential for the structural integrity and cofactor function of factor VIII. However, little information is available regarding how metal ion(s) fulfill these roles. Copper has been identified in the factor VIII heterodimer at a ratio of 1 mol/1 mol of factor VIII (*16*). Recently the reduced form of copper, Cu(I), has been identified in the protein using electron paramagnetic resonance (*19*). However, we show that neither Cu(I) nor Cu(II) reconstitutes cofactor activity from the isolated factor VIII subunits, whereas Ca(II) or Mn(II) support this reconstitution (*7*). Interestingly, the activity generated under these reconstitution conditions is less than the activity of the native material. This observation may be explained by an intrinsically lower specific activity of factor VIII due to the lack of an auxiliary component. While factor VIII treated with EDTA and subsequently dialyzed in Ca(II)-containing buffer yields similar specific activity material as compared with the factor VIII reconstituted from the isolated subunits, this activity is enhanced severalfold by either Cu(I) or Cu(II) ions and approaches the specific activity of native factor VIII (ref *19* and this study). Taken together, these results suggest two functional types of metal ion relative to factor VIII activity. One type [e.g., Mn(II) or Ca(II)] promotes generation of a "low specific activity" form of factor VIII; whereas copper ions [Cu(I) or Cu(II)] serve an auxiliary role to enhance activity of the preformed heterodimer.

Surprisingly, the presence of copper ions resulted in the inhibition of the Mn(II)- or Ca(II)-dependent reconstitution of factor VIII from isolated subunits. Preincubation of either subunit with Cu(II) revealed that this inhibitory activity was attributed to the factor VIII heavy chain, the likely overall effect of which was to reduce its affinity for the factor VIII light chain. Conversely, Cu(II) bound to factor VIII light chain resulted in an increased rate of reconstitution as well as a modest enhancement in reconstituted factor VIIIa activity. The reasons for these disparate effects produced by copper ions are not known. We speculate that a copper-dependent conformational change in the heavy chain results in its weakened affinity for light chain, whereas the increases in reaction rate of reassociation and in specific activity of factor VIII reflect a copper-dependent conformational change in the light chain.

Association of factor VIII heavy (A1-A2-B domains) and light (A3-C1-C2 domains) chains is metal ion-dependent with residues in the A1 and A3 domains containing the interactive sites. The observation that both Ca(II) and Mn(II) support this interaction is of interest since Ca(II) sites are typically formed by carboxylate moieties while Mn(II) can bind carboxylates as well as coordinated histidine residues (*29*). The divalent metal ion binding site(s) required for subunit association has (have) not been identified, and this topic remains controversial. A type II copper site has been proposed at the A1-A3 subunit interface using homology modeling, and occupancy of metal ion at this site is envisioned to bridge the heavy and light chains of factor VIII. A model based upon nitrite reductase (*17*) proposes that A1 subunit residues His^{99} and His^{161} with A3 subunit residue His^{1957} form this site. A more recent model (*18*) based upon the more homologous protein, ceruloplasmin, suggests that His^{161} is replaced by an oxygen atom of a water molecule H-bonded to the hydroxyl of Tyr^{106} and carbonyl of Ala^{100}. However, recent data showed that conversion of His^{1957} to Ala by site-directed mutagenesis yielded a factor VIII molecule that demonstrated wild type-like secretion, chain association, and activity, while conversion of His^{99} to Ala yielded a factor VIII with reduced chain association (*19*).

The above mutagenesis results, coupled with the capacity for Ca(II) to partially reconstitute factor VIII activity, suggest that this type II site may be dispensable for dimer formation. Thus, alternatives to a model wherein a divalent metal ion bridges the heavy and light chains of factor VIII (at this site) require consideration. For example, association of metal ion(s) with one or both chains may alter structure, resulting in increased interchain affinity and/or the active conformation. Additional results of Tagliavacca et al. (*19*) showed that conversion of Cys^{310} to Ser, a residue implicated in the proposed type I copper site of the A1 domain, yielded an inactive factor VIII partially defective for secretion while the heavy and light chains of the secreted protein were dissociated. These authors propose that this type I site coordinates Cu(I), providing an essential role for A1 domain folding that contributes to subsequent interaction with the A3 domain, whereas His^{99} is important for the intersubunit

interaction but not as a ligand for copper. Indeed, preliminary experiments using a solid-phase binding assay suggest a moderate affinity interaction ($K_d \sim 50$ nM) between the heavy and light chains in the absence of any divalent metal ion (Koshibu and Fay, unpublished observation). While this affinity is significantly weaker than that of the chains in the native factor VIII molecule ($K_d <$ nM), it suggests that a primary component of the interchain association is metal ion independent.

Our structural observations reveal that the conformations of both the heavy and light chain can be reversibly altered by divalent metal ions. Further, differential alterations in subunit conformation were observed in response to specific metals. Conformational analyses showed a gradient effect with no detectable alteration in secondary structure induced by Ca(II) (*28*), modest changes with Mn(II), and more dramatic changes in secondary structure with either Cu(I) or Cu(II). Extrinsic fluorescence analysis confirmed the disparate effects of the metal ions. In an earlier study, we used the apolar fluorophore (bis-ANS) to show that exposed hydrophobic sites on both the isolated factor VIII subunits likely participated in the intersubunit interaction (*28*). Here again, Cu(II) yielded a significant reduction in extrinsic fluorescence as compared with the reduction in fluorescence observed in the presence of Mn(II) or Ca(II). This reversible, metal ion-dependent reduction in fluorescence likely results from either direct displacement of the fluorophore and/or quenching. Furthermore, thermal denaturation profiles, as detected by the fluorescence of bound bis-ANS, indicated a marked difference between Ca(II) and Cu(II) or Mn(II). The latter ions showed similar low intensity biphasic thermal transitions whereas Ca(II) yielded a single transition. Thus, copper sites in factor VIII subunits are likely distinct from the sites that bind Mn(II) and/or Ca(II). This proposal is supported by the significant differences in subunit structures in response to the metal ions as well as the observation that the latter ions support subunit association while copper ions negatively and positively modulate the heavy and light chains, respectively. This modulation by copper may result indirectly following induction of a particular conformation and/or directly by contributing to (in the case of light chain) and/or blocking (in the case of heavy chain) an interactive site.

Copper effects on factor VIII structure and activity were minimal as compared with effects on the isolated subunits. We observed no Cu(II)-dependent effects on the CD spectra of factor VIII or on the fluorescence quenching/displacement of bis-ANS bound to the intact protein. These results likely reflect the presence of endogenous copper in the cofactor. Furthermore, the observed copper effects on bis-ANS fluorescence were likely specific for hydrophobic sites involved in heterodimer formation since the intersubunit interface is surface exposed on the subunits but not the intact protein. Factor VIII subunits were markedly sensitive to the metal ion. We observed that micromolar concentrations of copper altered the activities of the isolated factor VIII subunits, whereas millimolar concentrations were required to affect the activity of factor VIII. The mechanism by which relatively high levels of Cu(II) inactivate intact factor VIII is not clear but may occur by occupying weak affinity sites in the protein. Given the plasma concentration of Cu(II) ($\sim$20 μM; *29*), the interaction of the metal ion with intact protein is probably not physiologically important; whereas, the interaction of subunits with Cu(II) ($IC_{50} \sim 10$ μM) may be relevant.

A curious disparity exists when comparing the results of Tagliavacca et al. (*19*) using mutant factor VIII forms and our results using isolated subunits. The former study showed that alteration of the putative type I copper site in the heavy chain (Cys^{310} to Ser) resulted in inactive factor VIII and the secretion of dissociated chains; whereas alteration of the putative type I site in the light chain (Cys^{2000} to Ser) was benign. On the other hand, the present study suggests that copper binding to the heavy chain abrogates function, while its association with the light chain enhances reassociation rate and specific activity. There is currently insufficient information to reconcile these observations. One possible explanation is that the region in and around the copper site in the heavy chain is highly sensitive to alteration, either amino acid substitution or metal binding, and results in a conformational change that markedly reduces its affinity for light chain. Thus, copper may occupy a site in the light chain. Alteration of that site by mutagenesis may have only modest effect because of the apparent auxiliary role of copper in heterodimer formation.

The role of copper in factor VIII remains unknown, and we can only speculate about its function. Clearly, copper binding to the heavy chain is detrimental to function. Thus, some mechanism must exist to preclude association of the metal ion at this site. One possibility is that the site in heavy chain is masked by light chain residues and is inaccessible to copper. However, we observed that the association of copper with light chain has positive attributes. The copper-dependent acceleration of the intersubunit interaction modulated by the light chain could have implications in the proper folding of the single chain factor VIII precursor during its synthesis as well as the reconstitution of factor VIII following expression of separate chains (*30*). We also observed that association of Cu(II) enhanced the thermal stability of the light chain and the A1 subunit to greater extents than were observed with Ca(II). Thus, copper could contribute positively to the cofactor structure as well as activity. Indeed, this observation could help explain the physical basis for enhanced specific activity of the copper-containing molecule. Other potential roles for this metal ion in the biosynthesis and secretion of factor VIII have yet to be determined.

In summary, we have shown that copper ion serves an auxiliary role as a ligand in factor VIII. It does not directly contribute to heterodimer formation and, in fact, abrogates subunit association by modulating the heavy chain. However, when added to the preformed heterodimer, copper enhances specific activity severalfold and may contribute to the overall stability of the protein, likely by affecting the conformation of the light chain.

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