

## Exposed Hydrophobic Sites in Factor VIII and Isolated Subunits\*

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**Hydrophobic sites on the surface of factor VIII, factor VIIIA, and their derived subunits were evaluated using the fluorescent, apolar probe, bisanilinonaphthalsulfonic acid (bis-ANS). Two hydrophobic sites, with indicated affinities for the probe, were identified on factor VIII ( $K_d = 0.2$  and  $1.22 \mu\text{M}$ ), the isolated heavy chain (HC;  $K_d = 0.21$  and  $1.44 \mu\text{M}$ ), and light chain (LC;  $K_d = 0.04$  and  $0.22 \mu\text{M}$ ). Comparison of these values and fluorescence emission maxima parameters suggested that the higher affinity site on each isolated subunit contributes to the divalent metal ion-dependent, intersubunit interaction while the two lower affinity sites are retained on the surface of the factor VIII heterodimer. A single bis-ANS site was identified on both the A1/A3-C1-C2 dimer ( $K_d = 0.19 \mu\text{M}$ ) and A2 subunit ( $K_d = 0.11 \mu\text{M}$ ), whereas two sites, equivalent to the sites of factor VIII, were observed on factor VIIIA. These results suggested the absence of interactive hydrophobic sites between A2 subunit and dimer, a major conformational change around the hydrophobic site in A2 upon dissociation, and the lack of accessible hydrophobic regions on the B domain of HC.  $\text{Ca}^{2+}$  reduced the emission intensity of bis-ANS bound to the isolated LC, HC, and A1 subunit, but not the A2 subunit. Reconstitution of factor VIII activity from isolated HC and LC was inhibited by >90% in the presence of  $20 \mu\text{M}$  bis-ANS, whereas this concentration of probe had no effect on the reconstitution of FVIIIA from the A1/A3-C1-C2 dimer and A2 subunit. These results indicate that intersubunit hydrophobic interactions are important for the metal ion-dependent association between A1 and A3 domains, but are not required for the metal ion-independent interaction between A2 subunit and the A1/A3-C1-C2 dimer in factor VIIIA.**

Factor VIII, the plasma 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). Factor VIII is processed to a series of divalent metal ion-linked heterodimers (4–6) by cleavage at the A3-B junction, generating a HC<sup>1</sup> minimally represented by the A1-A2 domains, that may possess all or part of the B domain, and a LC consisting of the A3-C1-C2 domains. Factor VIII is inactivated by chelating reagents which dissociate the LC and HC (5, 6)

However, factor VIII activity can be reconstituted by combining the isolated subunits in the presence of  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$  (7, 8)

Factor VIII is proteolytically activated by thrombin to the active cofactor form, factor VIIIA. Thrombin cleaves the factor VIII at two sites within the HC, Arg<sup>740</sup> and Arg<sup>372</sup> (9). The former liberates the B domain or its fragments, while the latter bisects the A1 and A2 domains. Thrombin also cleaves at one site in the LC, at Arg<sup>1689</sup> (9), liberating an acidic region and creating a new N terminus. Thus factor VIIIA is a heterotrimer of A1, A2, and A3-C1-C2 subunits<sup>2</sup> (10, 11). The A1 subunit and the A3 domain retain the metal ion linkage and form a stable dimer designated A1/A3-C1-C2 (10). The A2 subunit associates with the dimer through primarily electrostatic interactions (10, 12). This weak affinity interaction ( $K_d \sim 260 \text{ nM}$  at physiological pH (13, 14)) is responsible for the observed lability of factor VIIIA. However, factor VIIIA activity can be reconstituted upon combining the isolated A1/A3-C1-C2 dimer and the A2 subunit at high relative subunit concentration and/or slightly acidic pH conditions (10, 12–14).

Little is known about structural elements in factor VIII and its activated form. Earlier results from our laboratory suggested that the reconstitution of factor VIII from isolated HC and LC was mediated by both electrostatic and hydrophobic interactions (8). In this report we identify and characterize the exposed hydrophobic sites on the surface of factor VIII, factor VIIIA, and isolated subunits using tryptophan fluorescence as well as extrinsic fluorescence from the apolar probe, bis-ANS. Results from this study indicate fewer exposed hydrophobic sites on factor VIII compared with isolated subunits, suggesting involvement of some of these sites in heterodimer formation. Additional analyses using factor VIIIA subunits permitted localization of these sites to factor VIII domains. Studies on the reconstitution of cofactor activity confirmed the importance of intersubunit hydrophobic interactions in the metal ion-dependent linkage within the factor VIII molecule.

### MATERIALS AND METHODS

**Reagents**—Recombinant factor VIII was generously provided by Debra Pittman of the Genetics Institute and by Dr. Jim Brown of the Bayer Corporation. Factor VIII subunits (15), factor VIIIA (16), factor VIIIA-derived A1/A3-C1-C2 dimer and A2 subunit (10), and A1 subunit (13) were prepared from recombinant factor VIII as described previously. Human  $\alpha$ -thrombin was obtained from Enzyme Research Laboratories. 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, 0.1 M NaCl at pH 7.0), filtered, and the concentration determined by absorbance at 385 nm using an extinction coefficient,  $\epsilon_{385} = 16,790 \text{ cm}^{-1} \text{ M}^{-1}$ .

**Assays**—Factor VIII activity was determined using a one stage clotting assay with substrate plasma that had been chemically depleted of factor VIII activity (17). Factor VIII was reconstituted from isolated HC

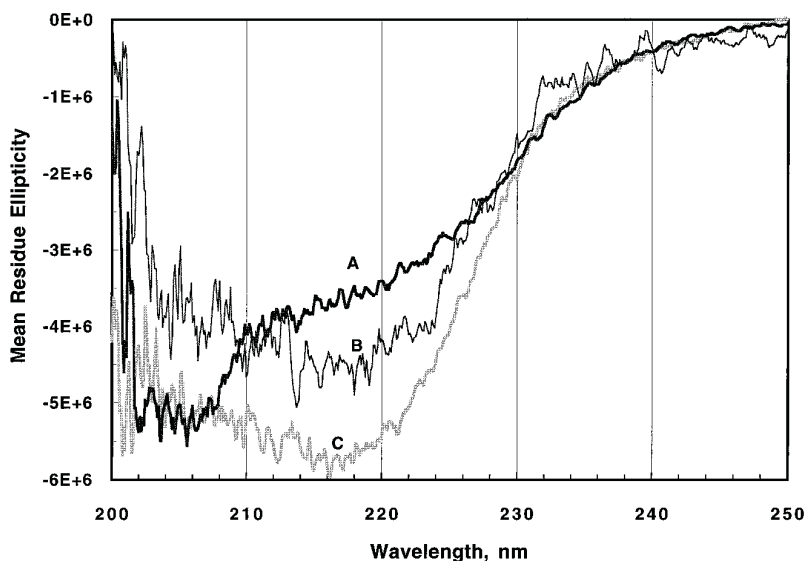
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<sup>1</sup> The abbreviations used are: HC, factor VIII heavy chain; LC, factor VIII light chain; bis-ANS, bisanilinonaphthalsulfonic acid; factor VIIIA, activated factor VIII; Mes, 4-morpholineethanesulfonic acid.

<sup>2</sup> Factor VIIIA subunits are designated relative to the domain sequence A1-A2-B-A3-C1-C2 (3) and are as follows: A1, residues 1–372; A2, residues 373–740; A3-C1-C2, residues 1690–2332. Noncovalent subunit associations are denoted by a slash (/) and covalent associations are denoted by a hyphen (-).

FIG. 1. CD spectra of HC (trace A), LC (trace B), and factor VIII (trace C). Sample contained 0.2  $\mu$ M protein in 0.02 M Tris and 0.1 M NaCl (pH 7). Spectra were recorded in a cylindrical fused quartz cell with a path length of 10 mm as described under "Materials and Methods." Mean residue ellipticities are expressed in degrees-cm<sup>2</sup>-dmol<sup>-1</sup>.



and LC (100 nM each) in a reaction containing 20 mM Tris (pH 7.2), 0.2 M NaCl, 0.01% Tween 20, and 20 mM MnCl<sub>2</sub>. Factor VIIIa reconstitution assays were performed using purified A1/A3-C1-C2 dimer and the A2 subunit (20 nM each) in 20 mM Mes (pH 6.0), 0.01% Tween 20, and 0.1 M NaCl. All reactions were run at room temperature. Other additions are as described in the figure legend. Protein concentrations were determined by the Coomassie Blue dye binding method of Bradford (1976) using bovine serum albumin as the standard.

**Fluorescence Spectroscopy**—Fluorescence measurements were performed using a SPEX Fluorolog 1681 spectrometer. The concentration of protein used for these studies was approximately 150 nM. The excitation and emission slit widths were set to 1.5 and 6 nm, 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 (18),  $F_{\text{corr}} = F_{\text{obs}} \cdot \text{antilog}(\text{OD}_{\text{ex}} + \text{OD}_{\text{em}}/2)$ , where  $\text{OD}_{\text{ex}}$  and  $\text{OD}_{\text{em}}$  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. Titration data of the bis-ANS-protein complex was analyzed by double-reciprocal plots (19, 20) or a least squares regression program (21, 22). The maximum dye to protein ratio for the complex formation was approximately 15–30. For the energy transfer experiments, an excitation wavelength of 295 nm was used, since this reduces or eliminates the involvement of tyrosine fluorescence from the protein. Data from the quenching of tryptophan emission by bis-ANS were analyzed using a modified Stern-Volmer equation (23). Resultant plots were used to calculate the  $K_d$  values of dye for the protein (24). The efficiency of energy transfer (EET) (25), from tryptophan to bis-ANS, was calculated from the formula,  $\text{EET} = 1 - (Q_d/Q_o)$ , where  $Q_d$  and  $Q_o$  are the relative fluorescence intensities of energy donor (tryptophan) in the presence and absence of energy acceptor (bis-ANS), respectively.

**Circular Dichroism Spectroscopy**—CD spectra in the range of 200–250 nm at room temperature were recorded on a Jasco-720 spectropolarimeter. Samples were measured in a cylindrical fused quartz cell with the path length of 10 mm, using a 2-nm bandwidth, 4-nm slit width, 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 200 nM in 20 mM Tris (pH 7.0) and 0.1 M NaCl. Observed ellipticities were converted to mean residue ellipticity and are expressed in degrees-cm<sup>2</sup>-dmol<sup>-1</sup>.

## RESULTS

**Secondary Structure of Factor VIII and Subunits**—The CD spectra of factor VIII is shown in Fig. 1. The negative band in the 210–220 nm region indicated the presence of a significant proportion of  $\beta$ -sheet content in the protein. The isolated HC and LC of factor VIII showed CD spectral properties similar to each other and to the parent factor VIII molecule (Fig. 1). That factor VIII and its subunits possess high  $\beta$ -sheet content is consistent with these molecules containing hydrophobic sites (26, 27). No additional conformational changes were detected in the secondary structure of either LC or HC upon the addition of

Ca<sup>2+</sup> or Mn<sup>2+</sup> (data not shown), suggesting that association of the subunits with divalent metal ions that allow for factor VIII reconstitution (7, 8) do not affect the gross structure of these subunits.

**Interaction of Bis-ANS with Factor VIII(a) and Subunits**—Exposed hydrophobic sites in factor VIII and its isolated subunits were probed with bis-ANS. In aqueous medium, this reagent shows an emission maximum at 533 nm (28). When bis-ANS binds to a hydrophobic moiety, its fluorescence intensity increases severalfold and the emission maxima are blue-shifted, the extent of which depends on the environment of the binding site (29, 30). A large increase in the bis-ANS fluorescence and a shift in the emission maximum from 533 to 498 nm were observed upon addition of a saturating level bis-ANS to factor VIII (Fig. 2). No further shift in the emission maximum was observed at higher concentrations of dye. In the absence of protein, the fluorescence intensity of 10  $\mu$ M bis-ANS in buffer was negligible compared with the fluorescence of dye in presence of protein (data not shown). Titration data yielded plots that were initially hyperbolic (up to approximately 0.4  $\mu$ M), but showed a further inflection at higher dye concentrations (Fig. 3). The shape of the titration curve indicated that bis-ANS had more than one binding site on the factor VIII. The  $K_d$  values calculated from these data are shown in Table I.

The isolated factor VIII subunits showed markedly different interactions with bis-ANS. In the presence of HC, low concentrations of bis-ANS (<0.4  $\mu$ M) showed an emission maximum at 489 nm (data not shown) that shifted slightly to a maximum of 492 nm upon further addition of dye (Fig. 2), indicating more than one binding site. Analysis of data showed the presence of two binding sites on HC. LC also showed similar results. These results suggest that both HC and LC possess two bis-ANS binding sites with different emission properties.  $K_d$  values (Table I) indicate that sites in the HC are of similar affinity to the sites in factor VIII, whereas the LC contains a higher affinity site as well as a site equivalent to the high affinity site in factor VIII or HC. The observation of two surface-exposed hydrophobic sites on each of the factor VIII subunits, as well as on the factor VIII heterodimer, indicates that the site(s) present on one or both of the isolated subunits participate in the intersubunit interaction and become inaccessible to bis-ANS.

Heterotrimeric factor VIIIa showed two bis-ANS sites (Table I) similar to the sites observed in factor VIII. This result indicated that the exposed hydrophobic sites were not localized to the B domain or the N-terminal region of the LC, since these regions are absent in the activated molecule. Factor VIIIa-

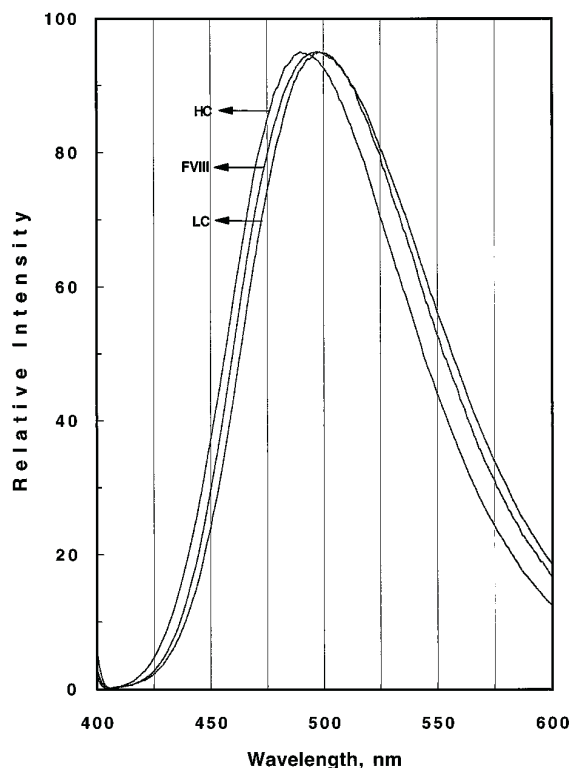


FIG. 2. Fluorescence emission spectra of bis-ANS in presence of factor VIII, HC, and LC. The excitation wavelength was 385 nm. The concentration of protein was 150 nM in 0.02 M Tris and 0.1 M NaCl (pH 7.0). Bis-ANS was at 10  $\mu$ M.

derived A1/A3-C1-C2 dimer and A2 subunit each showed a single binding site for bis-ANS with an emission maximum at 498 nm (data not shown). The affinity of dye for the two sites were similar to each other (Table I) and equivalent to the high affinity site observed on factor VIII. Evaluation of the isolated A1 subunit also revealed a single bis-ANS site that exhibited a lower affinity and reduced emission maximum compared with the site in A1/A3-C1-C2. That factor VIIIa contains two ANS sites, whereas single sites were observed on the A2 subunit and A1/A3-C1-C2 dimer, suggested that association of these components in factor VIIIa does not result from a hydrophobic-mediated interaction.

**Energy Transfer from Tryptophan to Bound Bis-ANS**—The affinities of bis-ANS for factor VIII and isolated HC and LC were also determined using fluorescence energy transfer. Energy transfer was detected from the overlap of the tryptophan fluorescence emission spectrum of factor VIII (or subunits) and excitation spectrum of bis-ANS. For these experiments, an excitation wavelength of 295 nm was used to avoid the interference from tyrosine fluorescence. Following addition of bis-ANS to factor VIII, there was a decrease in the tryptophan fluorescence emission at 339 nm concurrent with an increase in the bis-ANS fluorescence at 498 nm (Fig. 4A). The isoemissive point at 420 nm indicated that the binding sites of bis-ANS for factor VIII had the same emission spectra. We observed a slight deviation in the isoemissive point at 420 nm for the HC (Fig. 4B) and LC (data not shown). This result was consistent with the isolated subunits having binding sites for bis-ANS with different emission properties. Analysis of the tryptophan emission quenching data using a modified Stern-Volmer plot (23) was biphasic for factor VIII (Fig. 5) and HC and LC (data not shown), indicating the presence of two binding sites on the proteins. The  $K_d$  values obtained from this analysis were equivalent to those calculated from the simple titration data and are shown in Table I.

The efficiency of energy transfer from tryptophan to bound bis-ANS in factor VIII is shown in Table II. At saturating concentrations of bis-ANS, transfer efficiency reached a maximum of 40%. In factor VIIIa the transfer efficiency from tryptophan to bis-ANS was 45%. The similarity of these values was consistent with the above data, suggesting that the B domain did not participate in ANS binding. Energy transfer efficiency data observed for other subunits are also presented in Table II. In general, we observed that energy transfer appeared to be more efficient with the monomeric subunits (HC, LC, and A2) compared with dimeric/trimeric molecules (factor VIII, factor VIIIa, and A1/A3-C1-C2) and may reflect altered conformation in the hydrophobic sites when subunits are in the dissociated state.

**Effect of Bis-ANS on Factor VIII and Factor VIIIa Reconstitution**—In the presence of divalent metal ions, factor VIII can be reconstituted from isolated LC and HC (8), and this reconstitution was probed with bis-ANS. The probe inhibited the generation of clotting activity in a dose-dependent manner (Fig. 6). Less than 10% of maximal factor VIII activity was reconstituted in the presence of 20  $\mu$ M bis-ANS; whereas this concentration of bis-ANS had no effect on the clotting activity of native factor VIII (data not shown). Thus, hydrophobic sites on the LC and/or HC appear important for the  $\text{Me}^{2+}$ -dependent interaction between factor VIII subunits yielding functional cofactor, and these sites are effectively blocked by the probe. Contrary to the effect of bis-ANS on the reconstitution of factor VIII activity, relatively high concentrations of dye did not affect the reconstitution of factor VIIIa activity, consistent with the electrostatic nature of the association of A2 subunit with A1/A3-C1-C2 dimer (12, 13).

The extrinsic fluorescence of HC (100 nM) in the presence of an equimolar concentration of bis-ANS was evaluated for changes in fluorescence intensity following addition of  $\text{Me}^{2+}$  and LC (Fig. 7). Under these initial conditions, the probe should selectively occupy the high affinity site in HC. Addition of  $\text{CaCl}_2$  (20 mM) to the HC-dye complex resulted in a decrease in the fluorescence intensity without change in the peak position (489 nm), consistent with displacement of the dye by the  $\text{Ca}^{2+}$ . Subsequent addition of LC (100 nM) resulted in a further decrease in the fluorescence intensity without change in the emission maxima of dye-protein complex, suggesting that association of LC with HC resulted in a further displacement of the probe. Similar results were observed when the order of factor VIII subunits was reversed (data not shown). Furthermore,  $\text{Ca}^{2+}$  reduced the extrinsic fluorescence of the A1-dye complex, whereas  $\text{Ca}^{2+}$  had no effect on that of the A2-dye complex (data not shown). Taken together with the studies on reconstitution of factor VIII activity, these results suggest competition between bis-ANS and  $\text{Me}^{2+}$  for the high affinity sites in the subunits and indicate the importance of hydrophobic residues for  $\text{Me}^{2+}$  binding and regeneration of factor VIII activity.

**Effect of EDTA on Intrinsic Fluorescence of Factor VIII**—Further support for the participation of hydrophobic residues in the intersubunit interaction was obtained following changes in intrinsic fluorescence during dissociation of factor VIII. Addition of EDTA (10 mM) to factor VIII resulted in the decrease ( $\sim 20\%$ ) in the intrinsic fluorescence emission (recorded from 300 to 450 nm) of tryptophan residues in factor VIII with no detectable change in the wavelength of fluorescence emission maximum (Fig. 8). The excitation wavelength of 295 nm was used to avoid the energy transfer from tyrosines to tryptophans. This result suggested that metal ion chelation resulted in a change in the local environment of tryptophan residue(s), consistent with exposure of hydrophobic sites to the solvent.

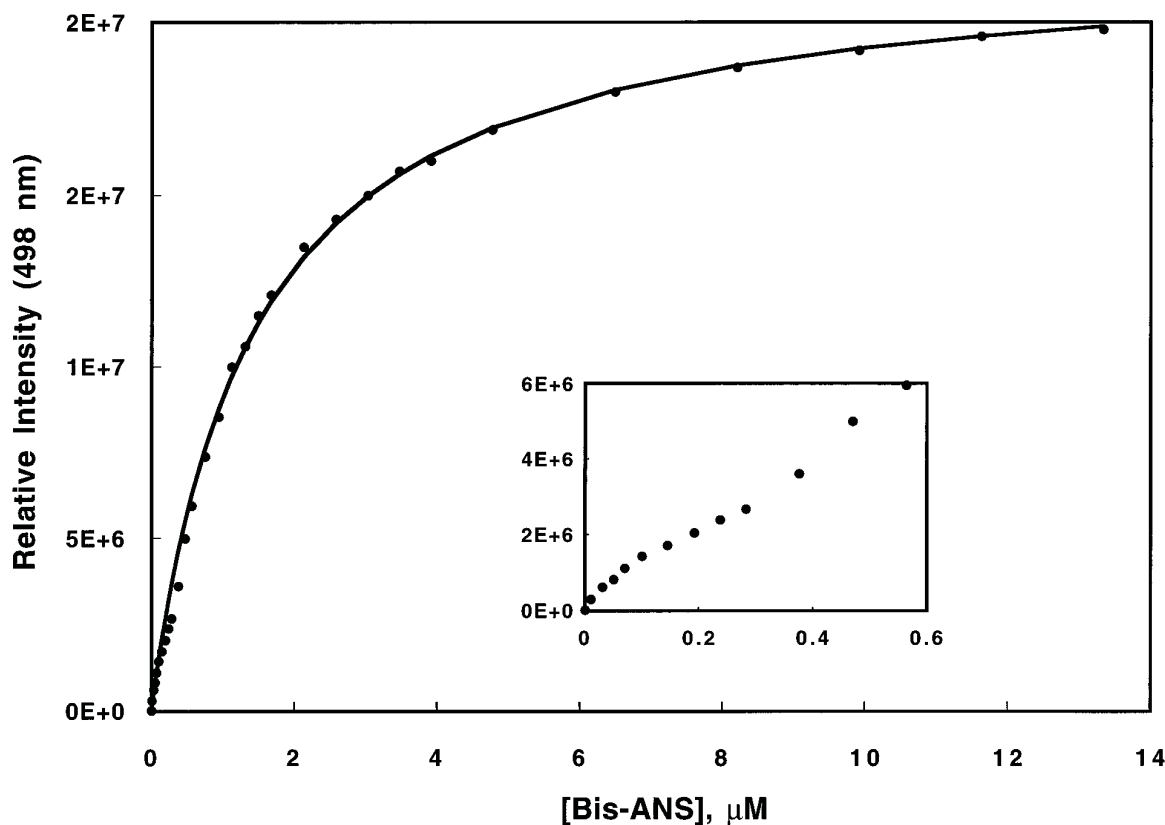


FIG. 3. **Titration of factor VIII at different concentrations of bis-ANS.** Factor VIII was at 0.2  $\mu\text{M}$ . The increase in bis-ANS fluorescence was monitored at a fixed emission wavelength of 498 nm. The excitation wavelength was 385 nm. The maximum dye/protein ratio for the complex formation is approximately 20.

TABLE I  
 $K_d$  values of bis-ANS for factor VIII and its subunits

Subunit	$\lambda_{\text{max}}$	$K_d^a$	$K_d^b$
	nm		$\mu\text{M}$
FVIII	498	0.20	0.19
	498	1.22	1.22
FVIIIa	498	0.20	0.20
	498	1.10	1.11
A1/A3-C1-C2	498	0.19	0.17
HC	489	0.21	0.21
	492	1.40	1.40
LC	492	0.04	0.04
	498	0.22	0.24
A1	492	0.77	0.75
A2	498	0.11	0.14

<sup>a</sup> Calculated from double-reciprocal plots.

<sup>b</sup> Calculated from energy transfer from Trp to bis-ANS data.

Furthermore, factor VIII, factor VIIIa, HC, and LC all showed emission maximum at 339 nm (data not shown), suggesting that most of the tryptophan residues are partially buried. Control experiments, performed in the same buffer, showed that EDTA did not quench the tryptophan fluorescence.

#### DISCUSSION

In this study we examine the role of exposed hydrophobic regions on factor VIII, factor VIIIa, and their isolated subunits in protein conformation, intersubunit interactions, and activity regeneration. Differential affinities of the apolar fluorophore, bis-ANS, for the protein substrates and resultant emission properties of the bound probe permitted the identification and localization of surface-exposed hydrophobic sites. The identical binding and emission properties of factor VIII and factor VIIIa for bis-ANS suggested the absence of hydrophobic sites on the factor VIII B domain. This conclusion was supported by the

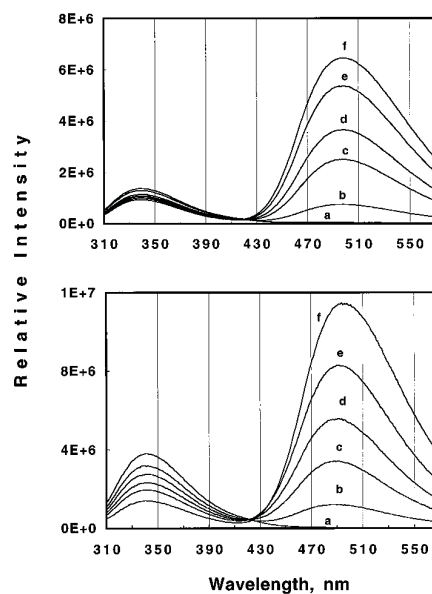


FIG. 4. **Energy transfer between the tryptophan residues and bound bis-ANS in factor VIII (upper panel) and HC (lower panel).** Excitation wavelength was 295 nm. Proteins were at 150 nM, and the added bis-ANS concentrations were 0 (trace a), 0.1 (trace b), 0.3 (trace c), 0.6 (trace d), 1.1 (trace e), and 3.0  $\mu\text{M}$  (trace f).

similarity of energy transfer efficiencies (Trp to bis-ANS) observed for factor VIII and factor VIIIa. The B domain, which exhibits significant heterogeneity in size (3) and is dispensable for clotting activity (6, 31), is characterized by electron microscopy as a rod-like tail structure extending up to 50 nm from a globular head, the latter comprised of the A and C domains (32). Thus the two hydrophobic sites identified on the isolated



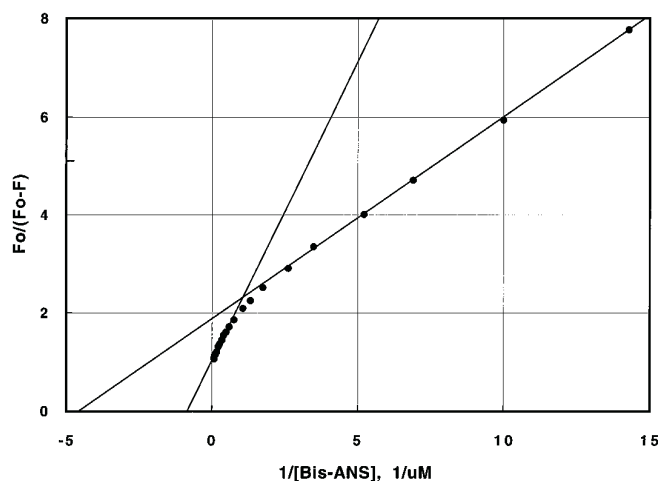


FIG. 5. Modified Stern-Volmer plot for the quenching of factor VIII intrinsic tryptophan fluorescence emission by bound bis-ANS. Fluorescence intensity of tryptophan emission was measured at 339 nm, and the excitation wavelength was 295 nm.

TABLE II

Efficiency of energy transfer from Trp to bound bis-ANS in factor VIII, factor VIIIa, and its isolated subunits

Subunits	Efficiency of energy transfer
	%
FVIII	40
FVIIIa	45
A1/A3-C1-C2	39
HC	60
LC	48
A1	37
A2	50

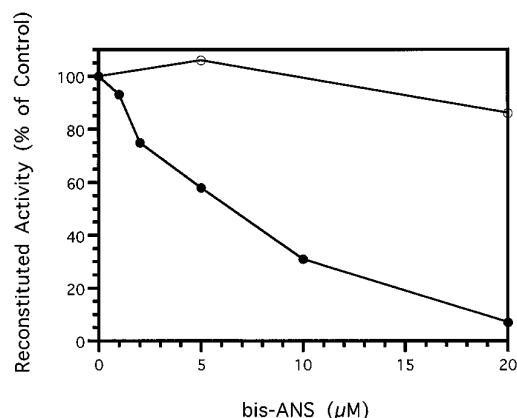


FIG. 6. Effect of bis-ANS on the reconstitution of factor VIII and factor VIIIa. Reconstitutions were performed as described under "Materials and Methods" in the presence of the indicated concentrations of bis-ANS. Activities are presented as the percent of the control reactions in the absence of fluorophore for factor VIII (closed circles) and factor VIIIa (open circles).

HC are contained within the A domains. These sites possess different emission properties, suggesting differences in the environment around them. Similarly, LC also showed two hydrophobic sites having different emission properties. Therefore, reconstitution of factor VIII from LC plus HC likely results in loss of two sites to the intersubunit interaction and retention of the remaining two sites on the surface of the factor VIII heterodimer. Based upon the similarity of the low affinity bis-ANS site on factor VIII ( $1.22 \mu\text{M}$ ) with that on the HC ( $1.4 \mu\text{M}$ ) and the identical fluorescence emission maxima (498 nm) values of the high affinity site on factor VIII ( $0.20 \mu\text{M}$ ) with the low affinity site on LC ( $0.22 \mu\text{M}$ ), we propose that these two hydro-

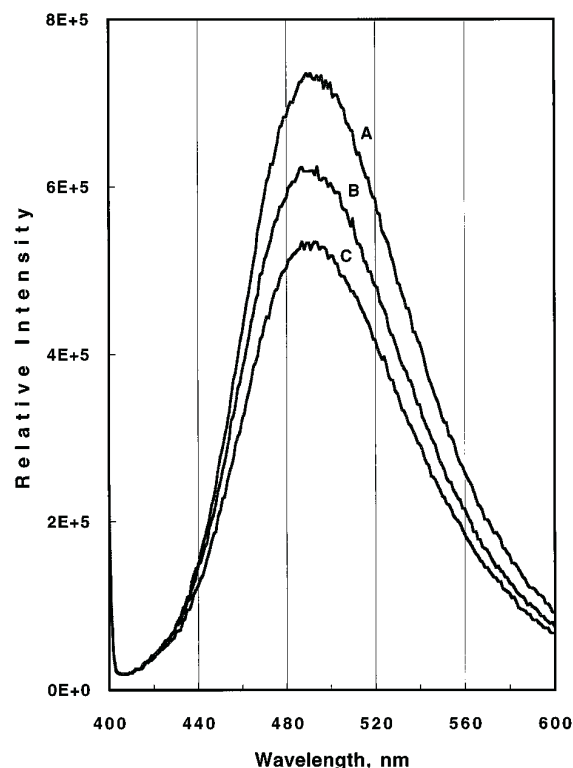


FIG. 7. Effect of  $\text{Ca}^{2+}$  and LC on the extrinsic fluorescence of bis-ANS-HC complex. Emission spectra of HC-bis-ANS complex alone in buffer (trace A), in the presence of  $\text{Ca}^{2+}$  (trace B), and subsequent addition of LC to spectrum B (trace C). The concentrations of HC, LC, and bis-ANS were at 100 nM each, and  $\text{CaCl}_2$  was at 20 mM in buffer containing 20 mM Tris and 0.1 M NaCl (pH 7.0). The excitation wavelength was 385 nm.

phobic sites on the free subunits are retained on the surface of factor VIII. Thus, we predict that the high affinity site present on HC ( $0.21 \mu\text{M}$ ) and on the LC ( $0.04 \mu\text{M}$ ) participate in the intersubunit association and as such are localized to the A1 domain and A3 domain, respectively.

Results obtained with the factor VIIIa-derived subunits were consistent with the above model. Interaction of the A1/A3-C1-C2 dimer with bis-ANS revealed a single binding site with a  $K_d$  of  $0.19 \mu\text{M}$ . We attribute this site to the LC-derived domains (A3-C1-C2), since its affinity and fluorescence properties are similar to that observed for the high affinity site on factor VIII ( $0.20 \mu\text{M}$ ). Therefore, the site observed on the isolated A1 subunit ( $0.77 \mu\text{M}$ ) likely contributes to the  $\text{Me}^{2+}$ -dependent interaction with A3-C1-C2 and is thus inaccessible in the dimer. This observation is consistent with the  $\text{Ca}^{2+}$ -dependent sensitivity of the bis-ANS site on A1 subunit. Thus the A2 domain, present in factor VIII but lacking in the A1/A3-C1-C2 dimer, likely contributes the weak affinity bis-ANS site ( $1.22 \mu\text{M}$ ) to factor VIII. Reconstitution of A1/A3-C1-C2 with A2 subunit to yield factor VIIIa resulted in the formation of two bis-ANS sites of equivalent affinities to those observed in the factor VIII molecule, confirming both the lack of hydrophobic sites on the B domain and the presence of a weak affinity site on the A2 subunit (domain). However, affinity of the fluorophore for the isolated A2 subunit was markedly (10-fold) greater ( $K_d = 0.11 \mu\text{M}$ ) when compared with values for the A2 subunit in association with the A1/A3-C1-C2 dimer ( $K_d = 1.10 \mu\text{M}$ ) or when the A2 domain is contiguous with the A1 domain in the factor VIII HC ( $K_d = 1.22 \mu\text{M}$ ). This result suggested an altered conformation around the hydrophobic site in A2 when free and/or that the site is partially buried and less accessible to the fluorophore when A2 is in complex.

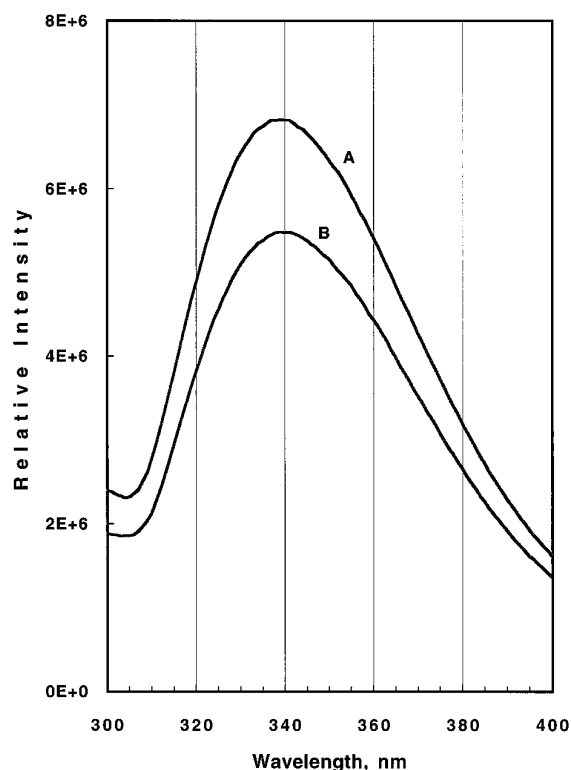


FIG. 8. Effect of EDTA on the intrinsic fluorescence of factor VIII. Emission spectra (excited at 295 nm) was obtained at room temperature for factor VIII (200 nM) in 20 mM Tris and 0.1 M NaCl (pH 7.0) in the absence (trace A) and presence (trace B) of 10 mM EDTA.

From the above model, both HC and LC contribute a single hydrophobic site in the formation of the factor VIII heterodimer. These sites may be involved in the binding of divalent metal ion based upon the observation that addition of  $\text{Ca}^{2+}$  to the complex of bis-ANS with HC or LC resulted in a decrease in fluorescence intensity. This result suggested that a fraction of the bound fluorophore was displaced by the metal ion. An alternative explanation, that the subunits were undergoing a metal ion-induced conformational change, which reduced the bis-ANS fluorescence, was not supported by our CD results or the observation that the fluorescence emission maxima remained unchanged. Furthermore, addition of either LC to HC-bis-ANS- $\text{Ca}^{2+}$  complex or HC to LC-bis-ANS- $\text{Ca}^{2+}$  complex resulted in a further decrease in the fluorescence intensity. Again, this result was consistent with displacement of fluorophore rather than alteration of conformation given the capacity of the apolar probe to block reconstitution of factor VIII. Taken together, these data support the role of hydrophobic interactions in the metal ion-dependent binding of HC and LC.

Intrinsic fluorescence and energy transfer studies further supported the role of hydrophobic residues in the inter-factor VIII subunit interaction. Factor VIII has 22 tryptophan residues in HC and 14 in the LC (3) that are randomly distributed in the subdomains of factor VIII. These residues show a fluorescence emission maximum at 339 nm, indicating that most of the tryptophans are partially buried (33). The observed decrease in the intrinsic tryptophan fluorescence on chelation of divalent metal with EDTA was likely the result of a major perturbation of environment around the tryptophan residues in the protein following the dissociation of HC and LC. This conformational change was also suggested by the observed increase in the efficiency of energy transfer (Trp to bis-ANS) for monomeric factor VIII(a) subunits compared with di- and trimeric factor VIII(a) forms.

A functional role for hydrophobic sites was confirmed following evaluation of the effects of bis-ANS on the reconstitution of factor VIII activity from isolated subunits. We observed that low concentrations of the probe resulted in marked inhibition of regeneration of factor VIII activity. In an earlier study (8), we showed that optimal reconstitution of factor VIII required moderate ionic strength, suggesting that both electrostatic and hydrophobic interactions were important for the reassociation of factor VIII subunits and regeneration of cofactor activity. We observed that bis-ANS had no effect on the activity of intact factor VIII, suggesting that the two exposed hydrophobic sites on the heterodimer either do not contribute to cofactor function or, if so, participate in high affinity interactions such that the probe is readily displaced by the competing macromolecule. The observation that bis-ANS had no effect on the reconstitution of factor VIIIa from the A1/A3-C1-C2 dimer and A2 subunit was consistent with this intersubunit interaction exclusively occurring by electrostatic forces (10, 12, 13).

Factor VIII A domains share approximately 40% sequence identity with the triplicated A domains of the circulating copper-binding protein, ceruloplasmin (3). Recent homology modeling studies (34) using several blue copper-binding proteins as a data base suggest the presence of a single type II copper-binding site in factor VIII. This putative site, formed from His<sup>99</sup> and His<sup>161</sup> of the A1 domain and His<sup>1957</sup> of the A3 domain, is believed to function in the divalent metal ion linkage between the HC and LC of factor VIII. The sequences around these His residues are: PVSLH<sup>99</sup>AVGV, SYLSH<sup>161</sup>VLDLV, and IHSIH<sup>1957</sup>FSGH (3). Inspection of these sequences reveals a high concentration of apolar residues, consistent with the participation of intersubunit hydrophobic interactions at or near the site of metal ion bonding. Furthermore, two tryptophan residues, Trp<sup>106</sup> and Trp<sup>1942</sup>, are located near His<sup>99</sup> and His<sup>1957</sup> in the linear sequence, respectively, and could serve as reporters for the metal ion-dependent changes in intrinsic fluorescence.

In summary, we present a model for the exposed hydrophobic sites in factor VIII and its isolated subunits. Structural and functional results indicate a role for a subset of these sites in the metal ion-dependent, inter-factor VIII subunit interaction required for expression of cofactor activity.

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